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(54) Title: POSH NUCLEIC ACIDS, POLYPEPTIDES AND RELATED METHODS

(57) Abstract: The application discloses novel polypeptides and nucleic acids involved in a variety of biological processes, including cellular proliferation. Related methods and compositions are also described.

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POSH NUCLEIC ACIDS, POLYPEPTIDES AND RELATED METHODS

RELATED APPLICATIONS

This application claims the benefit of the filing date of U.S. Provisional Application number 60/364,530, entitled "POSH nucleic acids, polypeptides and related methods" (filed March 15, 2002), the benefit of the filing date of U.S. Provisional Application number 60/391,629, entitled "PRT3 nucleic acids, polypeptides and related methods" (filed June 26, 2002), and the benefit of the filing date of U.S. Provisional Application number 60/429,916, entitled "POSH nucleic acids, polypeptides and related methods" (filed November 27, 2002). The entire teachings of the referenced Provisional Applications are incorporated herein by reference.

Background

Potential drug target validation involves determining whether a DNA, RNA or protein molecule is implicated in a disease process and is therefore a suitable target for development of new therapeutic drugs. Drug discovery, the process by which bioactive compounds are identified and characterized, is a critical step in the development of new treatments for human diseases. The landscape of drug discovery has changed dramatically due to the genomics revolution. DNA and protein sequences are yielding a host of new drug targets and an enormous amount of associated information.

The identification of genes and proteins involved in various disease states or key biological processes is a vital part of the drug design process. Many diseases and disorders could be treated or prevented by decreasing the expression of one or more genes involved in the molecular etiology of the condition if the appropriate molecular target could be identified and appropriate antagonists developed. For example, cancer, in which one or more cellular oncogenes become activated and result in the unchecked progression of cell cycle processes, could be treated by modulating appropriate cell cycle control genes. Furthermore many human genetic diseases, such as Huntington's disease, and certain prion conditions, which are influenced by both genetic and epigenetic factors, result from the inappropriate activity of a polypeptide as opposed to the complete loss of its function.

Accordingly, modulating the aberrant function of mutant genes would provide a means of treatment. Additionally, infectious diseases such as HIV have been successfully treated with molecular antagonists targeted to specific essential retroviral proteins such as HIV protease or reverse transcriptase. Drug therapy
5 strategies for treating such diseases and disorders have frequently employed molecular antagonists which target the polypeptide product of the disease gene(s). However the discovery of relevant gene or protein targets is often difficult and time consuming.

It is well known in the art that ubiquitin-mediated proteolysis is the major
10 pathway for the selective, controlled degradation of intracellular proteins in eukaryotic cells. Ubiquitin modification of a variety of protein targets within the cell appears to be important in a number of basic cellular functions such as regulation of gene expression, regulation of the cell-cycle, modification of cell surface receptors, biogenesis of ribosomes, and DNA repair. One major function of
15 the ubiquitin-mediated system is to control the half-lives of cellular proteins. The half-life of different proteins can range from a few minutes to several days, and can vary considerably depending on the cell-type, nutritional and environmental conditions, as well as the stage of the cell-cycle.

Targeted proteins undergoing selective degradation, presumably through the
20 actions of a ubiquitin-dependent proteasome, are covalently tagged with ubiquitin through the formation of an isopeptide bond between the C-terminal glycyl residue of ubiquitin and a specific lysyl residue in the substrate protein. This process is catalyzed by a ubiquitin-activating enzyme (E1) and a ubiquitin-conjugating enzyme (E2), and in some instances may also require auxiliary substrate recognition proteins
25 (E3s). Following the linkage of the first ubiquitin chain, additional molecules of ubiquitin may be attached to lysine side chains of the previously conjugated moiety to form branched multi-ubiquitin chains.

The conjugation of ubiquitin to protein substrates is a multi-step process. In an initial ATP requiring step, a thioester is formed between the C-terminus of
30 ubiquitin and an internal cysteine residue of an E1 enzyme. Activated ubiquitin is then transferred to a specific cysteine on one of several E2 enzymes. Finally, these E2 enzymes donate ubiquitin to protein substrates. Substrates are recognized either

directly by ubiquitin-conjugated enzymes or by associated substrate recognition proteins, the E3 proteins, also known as ubiquitin ligases.

It is also known that the ubiquitin system plays a role in a wide range of cellular processes including cell cycle progression, apoptosis, and turnover of many membrane receptors. The HIV Vpu protein interacts with an E3 protein that regulates I κ B degradation, and is thought to promote apoptosis of infected cells by indirectly inhibiting NF- κ B activity (Bour et al. (2001) J Exp Med 194:1299-311; U.S. Patent No. 5,932,425). The ubiquitin system regulates protein function by both mono-ubiquitination and poly-ubiquitination, and poly-ubiquitination is primarily associated with protein degradation.

The vesicular trafficking systems are the major pathways for the distribution of proteins among cell organelles, the plasma membrane and the extracellular medium. The vesicular trafficking systems may be directly or indirectly involved in a variety of disease states. The major vesicle trafficking systems in eukaryotic cells include those systems that are mediated by clathrin-coated vesicles and coatomer-coated vesicles. Clathrin-coated vesicles are generally involved in transport, such as in the case of receptor mediated endocytosis, between the plasma membrane and the early endosomes, as well as from the trans-Golgi network to endosomes. Coatomer-coated vesicles include coat protein I (COP-I) coated vesicles and COP-II coated vesicles, both of which tend to mediate transport of a variety of molecules between the ER and Golgi cisternae. In each case, a vesicle is formed by budding out from a portion of membrane that is coated with coat proteins, and the vesicle sheds its coat prior to fusing with the target membrane.

Clathrin coats assemble on the cytoplasmic face of a membrane, forming pits that ultimately pinch off to become vesicles. Clathrin itself is composed of two subunits, the clathrin heavy chain and the clathrin light chain, that form the clathrin triskelion. Clathrins associate with a host of other proteins, including the assembly protein, AP180, the adaptor complexes (AP1, AP2, AP3 and AP4), beta-arrestin, arrestin 3, auxilin, epsin, Eps15, v-SNAREs, amphiphysins, dynamin, synaptojanin and endophilin. The adaptor complexes promote clathrin cage formation, and help connect clathrin up to the membrane, membrane proteins, and many of the preceding components. AP1 associates with clathrin coated vesicles derived from the trans-

Golgi network and contains γ , $\beta 1$, $\mu 1$, and $\sigma 1$ polypeptide chains. AP2 associates with endocytic clathrin coated vesicles and contains α , $\beta 2$, $\mu 2$, and $\sigma 2$ polypeptides. Interactions between the clathrin complex and other proteins are mediated by a variety of domains found in the complex proteins, such as SH3 (Src homology 3) domains, PH (pleckstrin homology) domains, EH domains and NPF domains. (Marsh et al. (1999) Science 285:215-20; Pearse et al. (2000) Curr Opin Struct Biol 10(2):220-8).

Coatmer-coated vesicle formation is initiated by recruitment of a small GTPase (eg. ARF or SAR) by its cognate guanine nucleotide exchange factor (e.g. SEC12, GEA1, GEA2). The initial complex is recognized by a coat protein complex (COPI or COPII). The coat then grows across the membrane, and various cargo proteins become entrapped in the growing network. The membrane ultimately bulges and becomes a vesicle. The coat proteins stimulate the GTPase activity of the GTPase, and upon hydrolysis of the GTP, the coat proteins are released from the complex, uncoating the vesicle. Other proteins associated with coatmer coated vesicles include v-SNAREs, Rab GTPases and various receptors that help recruit the appropriate cargo proteins. (Springer et al. (1999) Cell 97:145-48).

It would be beneficial to identify proteins involved in one or more of these processes for use in, among other things, drug screening methods.

SUMMARY

In part, the application provides a novel ubiquitin ligase, POSH (Plenty Of SH3 domains) nucleic acid sequences and proteins encoded thereby. In certain embodiments, POSH polypeptides participate in cellular proliferation, such as, for example, a neoplastic condition, and in further embodiments, cellular proliferation may be influenced by manipulating a POSH activity. In additional embodiments, a neoplastic condition may be ameliorated in a subject by manipulating a POSH activity. In one embodiment, POSH polypeptides may stimulate ubiquitylation of certain proteins or stimulate membrane fusion or both. In certain embodiments, the invention relates to complexes comprising POSH. As one of skill in the art can readily appreciate, a POSH protein may form multiple different complexes at different times. In certain embodiments, the invention provides polypeptides that

associate with POSH (POSH AP) and polypeptides that involved in POSH mediated biological processes. In certain embodiments, a POSH polypeptide functions in membrane rearrangement and/or vesicular trafficking. In further embodiments, a POSH polypeptide regulates a Rac signaling pathway. In yet another embodiment, a POSH polypeptide regulates a JNK pathway. In an additional embodiment, a POSH polypeptide regulates NF-kB. In another embodiment, a POSH polypeptide regulates apoptosis.

In some aspects, the invention provides nucleic acid sequences and proteins encoded thereby, as well as oligonucleotides derived from the nucleic acid sequences, antibodies directed to the encoded proteins, screening assays to identify agents that modulate POSH, and diagnostic methods for detecting neoplastic cells and cells infected with a virus.

In one aspect, the invention provides an isolated nucleic acid comprising a nucleotide sequence which hybridizes under stringent conditions to a sequence of SEQ ID NOs: 1, 3, 4, 6, 8 and/or 10 or a sequence complementary thereto. In a related embodiment, the nucleic acid is at least about 80%, 90%, 95%, or 97-98%, or 100% identical to a sequence corresponding to at least about 12, at least about 15, at least about 25, at least about 40, at least about 100, at least about 300, at least about 500, at least about 1000, or at least about 2500 consecutive nucleotides up to the full length of SEQ ID NO: 1, 3, 4, 6, 8 and/or 10, or a sequence complementary thereto.

In one aspect, the invention provides an isolated nucleic acid comprising a nucleotide sequence which hybridizes under stringent conditions to a sequence of SEQ ID NOs: 31-35 or a sequence complementary thereto. In a related embodiment, the nucleic acid is at least about 80%, 90%, 95%, or 97-98%, or 100% identical to a sequence corresponding to at least about 12, at least about 15, at least about 25, consecutive nucleotides up to the full length of SEQ ID NO: 31-35, or a sequence complementary thereto.

In other embodiments, the invention provides a nucleic acid comprising a nucleotide sequence which hybridizes under stringent conditions to a sequence of SEQ ID Nos. 1, 3, 4, 6, 8 and/or 10, or a nucleotide sequence that is at least about 80%, 90%, 95%, or 97-98%, or 100% identical to a sequence corresponding to at

least about 12, at least about 15, at least about 25, at least about 40, at least about 100, at least about 300, at least about 500, at least about 1000, or at least about 2500 consecutive nucleotides up to the full length of SEQ ID NO: 1, 3, 4, 6, 8 and/or 10, or a sequence complementary thereto, and a transcriptional regulatory sequence
5 operably linked to the nucleotide sequence to render the nucleotide sequence suitable for use as an expression vector. In another embodiment, the nucleic acid may be included in an expression vector capable of replicating in a prokaryotic or eukaryotic cell. In a related embodiment, the invention provides a host cell transfected with the expression vector.

10 In yet another embodiment, the invention provides a substantially pure nucleic acid which hybridizes under stringent conditions to a nucleic acid probe corresponding to at least about 12, at least about 15, at least about 25, or at least about 40 consecutive nucleotides up to the full length of SEQ ID NO:1, 3, 4, 6, 8 and/or 10, or a sequence complementary thereto or up to the full length of the gene
15 of which said sequence is a fragment. The invention also provides an antisense oligonucleotide analog which hybridizes under stringent conditions to at least 12, at least 25, or at least 50 consecutive nucleotides up to the full length of SEQ ID NO:1 and/or 3, or a sequence complementary thereto.

In a further embodiment, the invention provides a nucleic acid comprising a
20 nucleic acid encoding an amino acid sequence as set forth in any of SEQ ID Nos: 2, 5, 7, 9 or 11, or a nucleic acid complement thereof. In a related embodiment, the encoded amino acid sequence is at least about 80%, 90%, 95%, or 97-98%, or 100% identical to a sequence corresponding to at least about 12, at least about 15, at least about 25, or at least about 40, at least about 100, at least about 200, at least about
25 300, at least about 400 or at least about 500 consecutive amino acids up to the full length of any of SEQ ID Nos:2, 5, 7, 9 or 11.

In another embodiment, the invention provides a probe/primer comprising a substantially purified oligonucleotide, said oligonucleotide containing a region of nucleotide sequence which hybridizes under stringent conditions to at least about 12,
30 at least about 15, at least about 25, or at least about 40 consecutive nucleotides of sense or antisense sequence selected from SEQ ID Nos: 1, 3, 4, 6, 8 and/or 10, or a sequence complementary thereto. In preferred embodiments, the probe selectively

hybridizes with a target nucleic acid. In another embodiment, the probe may include a label group attached thereto and able to be detected. The label group may be selected from radioisotopes, fluorescent compounds, enzymes, and enzyme co-factors. The invention further provides arrays of at least about 10, at least about 25,
5 at least about 50, or at least about 100 different probes as described above attached to a solid support.

In another aspect, the invention provides polypeptides. In one embodiment, the invention pertains to a polypeptide including an amino acid sequence encoded by a nucleic acid comprising a nucleotide sequence which hybridizes under stringent
10 conditions to a sequence of SEQ ID Nos:1, 3, 4, 6, 8 and/or 10, or a sequence complementary thereto, or a fragment comprising at least about 25, or at least about 40 amino acids thereof.

In a preferred embodiment, the POSH polypeptide comprises a sequence that is identical with or homologous to any of SEQ ID Nos: 2, 5, 7, 9 or 11. For
15 instance, a POSH polypeptide preferably has an amino acid sequence at least 60% homologous to a polypeptide represented by any of SEQ ID Nos:2, 5, 7, 9 or 11 and polypeptides with higher sequence homologies of, for example, 80%, 90% or 95% are also contemplated. The POSH polypeptide can comprise a full length protein, such as represented in the sequence listings, or it can comprise a fragment of, for
20 instance, at least 5, 10, 20, 50, 100, 150, 200, 250, 300, 400 or 500 or more amino acids in length.

In another embodiment, the application provides polypeptides comprising a sequence that is at least 80%, 90% or 95% identical with or homologous to any of SEQ ID Nos: 26-30.

25 In another preferred embodiment, the invention features a purified or recombinant polypeptide fragment of a POSH polypeptide, which polypeptide has the ability to modulate, e.g., mimic or antagonize, an activity of a wild-type POSH protein. Preferably, the polypeptide fragment comprises a sequence identical or homologous to an amino acid sequence designated in any of SEQ ID Nos: 2, 5, 7, 9
30 or 11.

Moreover, as described below, the POSH polypeptide can be either an agonist (e.g. mimics), or alternatively, an antagonist of a biological activity of a

naturally occurring form of the protein, e.g., the polypeptide is able to modulate the intrinsic biological activity of a POSH protein or a POSH complex, such as an enzymatic activity, binding to other cellular components, cellular compartmentalization, membrane reorganization and the like.

- 5 The subject proteins can also be provided as chimeric molecules, such as in the form of fusion proteins. For instance, the POSH polypeptide can be provided as a recombinant fusion protein which includes a second polypeptide portion, e.g., a second polypeptide having an amino acid sequence unrelated (heterologous) to POSH, e.g. the second polypeptide portion is glutathione-S-transferase, e.g. the
10 second polypeptide portion is an enzymatic activity such as alkaline phosphatase, e.g. the second polypeptide portion is an epitope tag, etc.

- Yet another aspect of the present invention concerns an immunogen comprising a POSH polypeptide in an immunogenic preparation, the immunogen being capable of eliciting an immune response specific for the POSH polypeptide;
15 e.g. a humoral response, e.g. an antibody response; e.g. a cellular response. In preferred embodiments, the immunogen comprises an antigenic determinant, e.g. a unique determinant, from a protein represented by SEQ ID NO:2.

- In yet another aspect, this invention provides antibodies immunoreactive with one or more POSH polypeptides. In one embodiment, antibodies are specific
20 for an SH3 domain or a RING domain derived from a POSH polypeptide. In a more specific embodiment, the domain is part of an amino acid sequence set forth in SEQ ID NO:2. In a set of exemplary embodiments, an antibody binds to one or more SH3 domains represented by amino acids 137-192, 199-258, 448-505 and 832-888 of SEQ ID NO:2 and are set forth in any one of SEQ ID Nos: 27-30. In another
25 exemplary embodiment, an antibody binds to a RING domain represented by amino acids 12-52 of SEQ ID NO:2 and is set forth in SEQ ID No: 26. In another embodiment, the antibodies are immunoreactive with one or more proteins having an amino acid sequence that is at least 80% identical, at least 90% identical or at least 95% identical to an amino acid sequence as set forth in SEQ ID NO:2. In other
30 embodiments, an antibody is immunoreactive with one or more proteins having an amino acid sequence that is 85%, 90%, 95%, 98%, 99% or identical to an amino acid sequence as set forth in SEQ ID NO:2.

In certain embodiments, the subject POSH nucleic acids will include a transcriptional regulatory sequence, e.g. at least one of a transcriptional promoter or transcriptional enhancer sequence, which regulatory sequence is operably linked to the POSH sequence. Such regulatory sequences can be used to render the POSH sequence suitable for use as an expression vector.

In yet another aspect, the invention provides an assay for screening test compounds for inhibitors, or alternatively, potentiators, of an interaction between a POSH polypeptide and a POSH-associated protein (POSH-AP). An exemplary method includes the steps of (i) combining POSH-AP, a POSH polypeptide, and a test compound, e.g., under conditions wherein, but for the test compound, the POSH polypeptide and POSH-AP are able to interact; and (ii) detecting the formation of a complex which includes the POSH polypeptide and a POSH-AP. A statistically significant change, such as a decrease, in the formation of the complex in the presence of a test compound (relative to what is seen in the absence of the test compound) is indicative of a modulation, e.g., inhibition, of the interaction between the POSH polypeptide and POSH-AP.

In a further embodiment, the invention provides an assay for identifying a test compound which inhibits or potentiates the interaction of a POSH polypeptide to a POSH-AP, comprising (a) forming a reaction mixture including POSH polypeptide, a POSH-AP; and a test compound; and detecting binding of said POSH polypeptide to said POSH-AP; wherein a change in the binding of said POSH polypeptide to said POSH-AP in the presence of the test compound, relative to binding in the absence of the test compound, indicates that said test compound potentiates or inhibits binding of said POSH polypeptide to said POSH-AP.

In additional embodiment, the invention relates to a method for identifying modulators of protein complexes, comprising (a) forming a reaction mixture comprising a POSH polypeptide, a POSH-AP; and a test compound; (b) contacting the reaction mixture with a test agent, and (c) determining the effect of the test agent for one or more activities. Exemplary activities include a change in the level of the protein complex, a change in the enzymatic activity of the complex, where the reaction mixture is a whole cell, a change in the plasma membrane localization of

the complex or a component thereof or a change in the interaction between the POSH polypeptide and the POSH-AP.

An additional embodiment is a screening assay to identify agents that inhibit or potentiate the interaction of a POSH polypeptide and a POSH-AP, comprising
5 providing a two-hybrid assay system including a first fusion protein comprising a POSH polypeptide portion of SEQ ID NO:2, and a second fusion protein comprising a POSH-AP portion, under conditions wherein said two hybrid assay is sensitive to interactions between the POSH polypeptide portion of said first fusion protein and said POSH-AP portion of said second polypeptide; measuring a level of interactions
10 between said fusion proteins in the presence and in the absence of a test agent; and comparing the level of interaction of said fusion proteins, wherein a decrease in the level of interaction is indicative of an agent that will inhibit the interaction between a POSH polypeptide and a POSH-AP.

In additional aspects, the invention provides isolated protein complexes
15 including a combination of a POSH polypeptide and at least one POSH-AP. In certain embodiments, a POSH complex is related to clathrin-coated vesicle formation. In certain embodiments, a POSH complex relates to a ubiquitin related activity of POSH, as in the case of POSH complexes comprising ubiquitin (e.g., covalent or non-covalent POSH ubiquitin conjugates), an E2, an E1 or a
20 ubiquitination target.

In an additional aspect, the invention provides nucleic acid therapies for manipulating POSH. In one embodiment, the invention provides a ribonucleic acid comprising between 5 and 1000 consecutive nucleotides of a nucleic acid sequence that is at least 90%, 95%, 98%, 99% or optionally 100% identical to a sequence of
25 SEQ ID NO:1 and/or 3 or a complement thereof. Optionally the ribonucleic acid comprises at least 10, 15, 20, 25, or 30 consecutive nucleotides, and no more than 1000, 750, 500 and 250 consecutive nucleotides of a POSH nucleic acid. In certain embodiments the ribonucleic acid is an RNAi oligomer or a ribozyme. Preferably, the ribonucleic acid decreases the level of a POSH mRNA. Preferred ribonucleic
30 acids comprise a sequence selected from any of SEQ ID Nos: 15, 16, 18, 19, 21, 22, 24 and 25.

The invention also features transgenic non-human animals, e.g. mice, rats, rabbits, goats, sheep, dogs, cats, cows, or non-human primates, having a transgene, e.g., animals which include (and preferably express) a heterologous form of the POSH gene described herein. Such a transgenic animal can serve as an animal
5 model for studying neoplastic conditions or for use in drug screening for treating neoplastic conditions.

In further aspects, the invention provides compositions for the delivery of a nucleic acid therapy, such as, for example, compositions comprising a liposome and/or a pharmaceutically acceptable excipient or carrier.

10 The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook,
15 Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); *DNA Cloning*, Volumes I and II (D. N. Glover ed., 1985); *Oligonucleotide Synthesis* (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss,
20 Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); *Methods In Enzymology*, Vols. 154 and 155 (Wu et al. eds.), *Immunochemical Methods In Cell*
25 *And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Other features and advantages of the invention will be apparent from the
30 following detailed description, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

- Figure 1: Human POSH Coding Sequence (SEQ ID NO:1)
- Figure 2: Human POSH Amino Acid Sequence (SEQ ID NO:2)
- Figure 3: Human POSH cDNA Sequence (SEQ ID NO:3)
- Figure 4: 5' cDNA fragment of human POSH (public gi:10432611; SEQ ID NO:4)
- 5 Figure 5: N terminus protein fragment of hPOSH (public gi:10432612; SEQ ID NO:5)
- Figure 6: 3' mRNA fragment of hPOSH (public gi:7959248; SEQ ID NO:6)
- Figure 7: C terminus protein fragment of hPOSH (public gi:7959249; SEQ ID NO:7)
- 10 Figure 8: Human POSH full mRNA, annotated sequence
- Figure 9: Domain analysis of human POSH
- Figure 10: Diagram of human POSH nucleic acids. The diagram shows the full-length POSH gene and the position of regions amplified by RT-PCR or targeted by siRNA used in figure 11.
- 15 Figure 11: Knockdown of POSH mRNA by siRNA duplexes. HeLa SS-6 cells were transfected with siRNA against Lamin A/C (lanes 1, 2) or POSH (lanes 3-10). POSH siRNA was directed against the coding region (153 - lanes 3,4; 155 - lanes 5,6) or the 3'UTR (157 - lanes 7, 8; 159 - lanes 9, 10). Cells were harvested 24 hours post-transfection, RNA extracted, and POSH mRNA levels compared by RT-PCR of
- 20 a discrete sequence in the coding region of the POSH gene (see figure 10). GAPDH is used as an RT-PCR control in each reaction.
- Figure 12: Reduction of POSH levels reversibly inhibits HeLa cell growth.
- Figure 13: Expression of POSH protein. A. Expression profile of POSH in different cell lines.
- 25 B. Tissue-specific expression of POSH protein.
- Figure 14: Mouse POSH mRNA sequence (public gi:10946921; SEQ ID NO: 8)
- Figure 15: Mouse POSH Protein sequence (Public gi: 10946922; SEQ ID NO: 9)
- Figure 16: Drosophila melanogaster POSH mRNA sequence (public gi:17737480; SEQ ID NO:10)
- 30 Figure 17: Drosophila melanogaster POSH protein sequence (public gi:17737481; SEQ ID NO:11)
- Figure 18: POSH Domain Analysis

Figure 19: POSH Expression in thyroid carcinoma as shown by immunohistochemistry of human thyroid sections. Top: thyroid carcinoma Bottom: normal thyroid gland.

Figure 20: POSH Expression in tumors as shown by immunohistochemistry of human tumor tissue sections: A. Lymphoma B. osteosarcoma C. liposarcoma D. normal lung (left panel), lung carcinoma (right panel).

Figure 21: Human POSH has ubiquitin ligase activity

Figure 22: Human POSH co-immunoprecipitates with RAC1

Figure 23: Human POSH is localized at two sites: one at the nucleus and one at the golgi (C) . After HIV transfection POSH golgi localization is enhanced(D). HeLa and 293T cells were transfected with pNLenv-1. 24 hours post transfection the cells were incubated with primary antibodies against POSH and either with anti-p24 Gag (B) or an organelle marker as follows: BiP- endoplasmatic reticulum (data not shown), GM130- golgi, (C,D) nucleoporin- nuclei matrix (E, F) and histone H1- nucleus (data not shown). Two different fluorescence labeled secondary antibodies were used. Specific signals were obtained by laser-scanning confocal microscopy, and individual signals were overlaid to assess their relative positions. POSH is localized to the nucleus (A) and partially colocalized with HIV-1 Gag and the golgi marker outside the nucleus, presumably in the nuclear matrix (B) following transfection..

DETAILED DESCRIPTION OF THE INVENTION

1. Definitions

The articles "a" and "an" are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

"Biological samples" refers to any tissue or liquid sample having genomic DNA or other nucleic acids (e.g. mRNA) or proteins. It includes both cells with a normal complement of chromosomes and cells suspected of neoplastic conditions.

The term "binding" refers to a direct association between two molecules, due to, for example, covalent, electrostatic, hydrophobic, ionic and/or hydrogen-bond interactions under physiological conditions.

"Cells," "host cells" or "recombinant host cells" are terms used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either
5 mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

A "chimeric protein" or "fusion protein" is a fusion of a first amino acid sequence encoding a polypeptide with a second amino acid sequence defining a domain foreign to and not substantially homologous with any domain of the first
10 amino acid sequence. A chimeric protein may present a foreign domain which is found (albeit in a different protein) in an organism which also expresses the first protein, or it may be an "interspecies", "intergenic", etc. fusion of protein structures expressed by different kinds of organisms.

The terms "compound", "test compound" and "molecule" are used herein
15 interchangeably and are meant to include, but are not limited to, peptides, nucleic acids, carbohydrates, small organic molecules, natural product extract libraries, and any other molecules (including, but not limited to, chemicals, metals and organometallic compounds).

The terms "comprise" and "comprising" are used in the inclusive, open
20 sense, meaning that additional elements may be included.

The phrase "conservative amino acid substitution" refers to grouping of amino acids on the basis of certain common properties. A functional way to define common properties between individual amino acids is to analyze the normalized frequencies of amino acid changes between corresponding proteins of homologous
25 organisms (Schulz, G. E. and R. H. Schirmer., Principles of Protein Structure, Springer-Verlag). According to such analyses, groups of amino acids may be defined where amino acids within a group exchange preferentially with each other, and therefore resemble each other most in their impact on the overall protein structure (Schulz, G. E. and R. H. Schirmer., Principles of Protein Structure,
30 Springer-Verlag). Examples of amino acid groups defined in this manner include:

- (i) a charged group, consisting of Glu and Asp, Lys, Arg and His,
- (ii) a positively-charged group, consisting of Lys, Arg and His,

- (iii) a negatively-charged group, consisting of Glu and Asp,
- (iv) an aromatic group, consisting of Phe, Tyr and Trp,
- (v) a nitrogen ring group, consisting of His and Trp,
- (vi) a large aliphatic nonpolar group, consisting of Val, Leu and Ile,
- 5 (vii) a slightly-polar group, consisting of Met and Cys,
- (viii) a small-residue group, consisting of Ser, Thr, Asp, Asn, Gly, Ala, Glu, Gln and Pro,
- (ix) an aliphatic group consisting of Val, Leu, Ile, Met and Cys, and
- (x) a small hydroxyl group consisting of Ser and Thr.

10 In addition to the groups presented above, each amino acid residue may form its own group, and the group formed by an individual amino acid may be referred to simply by the one and/or three letter abbreviation for that amino acid commonly used in the art.

A "conserved residue" is an amino acid that is relatively invariant across a
15 range of similar proteins. Often conserved residues will vary only by being replaced with a similar amino acid, as described above for "conservative amino acid substitution".

The term "domain" as used herein refers to a region of a protein that comprises a particular structure and/or performs a particular function.

20 "Homology" or "identity" or "similarity" refers to sequence similarity between two peptides or between two nucleic acid molecules. Homology and identity can each be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When an equivalent position in the compared sequences is occupied by the same base or amino acid, then the molecules
25 are identical at that position; when the equivalent site occupied by the same or a similar amino acid residue (e.g., similar in steric and/or electronic nature), then the molecules can be referred to as homologous (similar) at that position. Expression as a percentage of homology/similarity or identity refers to a function of the number of identical or similar amino acids at positions shared by the compared sequences. A
30 sequence which is "unrelated" or "non-homologous" shares less than 40% identity, though preferably less than 25% identity with a sequence of the present invention.

In comparing two sequences, the absence of residues (amino acids or nucleic acids) or presence of extra residues also decreases the identity and homology/similarity.

The term "homology" describes a mathematically based comparison of sequence similarities which is used to identify genes or proteins with similar functions or motifs. The nucleic acid and protein sequences of the present invention may be used as a "query sequence" to perform a search against public databases to, for example, identify other family members, related sequences or homologs. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (1990) J Mol. Biol. 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score=100, wordlength=12 to obtain nucleotide sequences homologous to nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score=50, wordlength=3 to obtain amino acid sequences homologous to protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Res. 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and BLAST) can be used. See <http://www.ncbi.nlm.nih.gov>.

As used herein, "identity" means the percentage of identical nucleotide or amino acid residues at corresponding positions in two or more sequences when the sequences are aligned to maximize sequence matching, i.e., taking into account gaps and insertions. Identity can be readily calculated by known methods, including but not limited to those described in (Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48: 1073 (1988). Methods to determine identity are designed to give the largest match between the sequences tested. Moreover, methods to determine identity are codified in publicly available computer programs. Computer

program methods to determine identity between two sequences include, but are not limited to, the GCG program package (Devereux, J., et al., Nucleic Acids Research 12(1): 387 (1984)), BLASTP, BLASTN, and FASTA (Altschul, S. F. et al., J. Molec. Biol. 215: 403-410 (1990) and Altschul et al. Nuc. Acids Res. 25: 3389-3402
5 (1997)). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, Md. 20894; Altschul, S., et al., J. Mol. Biol. 215: 403-410 (1990). The well known Smith Waterman algorithm may also be used to determine identity.

The term "including" is used herein to mean "including but not limited to".
10 "Including" and "including but not limited to" are used interchangeably.

The term "intron" refers to a portion of nucleic acid that is initially transcribed into RNA but later removed such that it is not, for the most part, represented in the processed mRNA. Intron removal occurs through reactions at the 5' and 3' ends, typically referred to as 5' and 3' splice sites, respectively. Alternate
15 use of different splice sites results in splice variants. An intron is not necessarily situated between two "exons", or portions that code for amino acids, but may instead be positioned, for example, between the promoter and the first exon. An intron may be self-splicing or may require cellular components to be spliced out of the mRNA. A "heterologous intron" is an intron that is inserted into a coding sequence that is
20 not naturally associated with that coding sequence. In addition, a heterologous intron may be a generally natural intron wherein one or both of the splice sites have been altered to provide a desired quality, such as increased or decreased splice efficiency. Heterologous introns are often inserted, for example, to improve expression of a gene in a heterologous host, or to increase the production of one
25 splice variant relative to another. As an example, the rabbit beta-globin gene may be used, and is commercially available on the pCI vector from Promega Inc. Other exemplary introns are provided in Lacy-Hulbert et al. (2001) Gene Ther 8(8):649-53.

The term "isolated", as used herein with reference to the subject proteins and
30 protein complexes, refers to a preparation of protein or protein complex that is essentially free from contaminating proteins that normally would be present with the protein or complex, e.g., in the cellular milieu in which the protein or complex is

found endogenously. Thus, an isolated protein complex is isolated from cellular components that normally would "contaminate" or interfere with the study of the complex in isolation, for instance while screening for modulators thereof. It is to be understood, however, that such an "isolated" complex may incorporate other proteins the modulation of which, by the subject protein or protein complex, is being investigated.

The term "isolated" as also used herein with respect to nucleic acids, such as DNA or RNA, refers to molecules in a form which does not occur in nature. Moreover, an "isolated nucleic acid" is meant to include nucleic acid fragments which are not naturally occurring as fragments and would not be found in the natural state.

As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single-stranded (such as sense or antisense) and double-stranded polynucleotides.

The term "maturation" as used herein refers to the production, post-translational processing, assembly and/or release of proteins that form a viral particle. Accordingly, this includes the processing of viral proteins leading to the pinching off of nascent virion from the cell membrane.

A "membrane associated protein" is meant to include proteins that are integral membrane proteins as well as proteins that are stably associated with a membrane.

A "neoplastic cell" is a cell that exhibits a tendency to proliferate that is recognizably greater than normal. Exemplary neoplastic cells include immortalized cell lines, hyperproliferative cells cultured from a neoplasm, cells transfected with, for example, an oncogene to promote growth, etc.

A "neoplastic condition" includes any disease, disorder, symptom or other bodily condition, whether or not it is detrimental to health, that involves cells proliferating to a degree that is recognizably greater than normal. Neoplastic

conditions include benign growths, such as warts, as well as benign and malignant tumors and precancerous growths.

The term “or” as used herein should be understood to mean “and/or”, unless the context clearly indicates otherwise.

5 The term “proliferate” as used in reference to cells means to increase in number. Increases in cellular proliferation as seen at the population level may result from a variety of causes, including more rapid cell division or a decreased rate of cell death, or a mixture. Decreases in cellular proliferation as seen at the population level may similarly result from a variety of causes.

10 A “POSH nucleic acid” is a nucleic acid comprising a sequence as represented in any of SEQ ID Nos:1, 3, 4, 6, 8, and 10 as well as any of the variants described herein.

 A “POSH polypeptide” or “POSH protein” is a polypeptide comprising a sequence as represented in any of SEQ ID Nos: 2, 5, 7, 9 and 11 as well as any of the variations described herein.

15 A “POSH-associated protein” or “POSH-AP” refers to a protein capable of interacting with and/or binding to a POSH polypeptide. Generally, the POSH-AP may interact directly or indirectly with the POSH polypeptide. Exemplary POSH-APs are provided throughout.

20 The terms peptides, proteins and polypeptides are used interchangeably herein.

 A “profile” is used herein to indicate an aggregate of information regarding a preparation of cell or membrane surface proteins. A profile will comprise, at minimum, information regarding the presence or absence of such proteins. More typically, a profile will comprise information regarding the presence or absence of a plurality of such proteins. In addition, a profile may contain other information about each identified protein, such as relative or absolute amount of protein present, the degree of post-translational modification, membrane topology, three-dimensional structure, isoelectric point, molecular weight, etc. A “test profile” is a profile obtained from a subject of unknown diagnostic state. A “reference profile” is a profile obtained from subject known to be infected or uninfected.

The term "purified protein" refers to a preparation of a protein or proteins which are preferably isolated from, or otherwise substantially free of, other proteins normally associated with the protein(s) in a cell or cell lysate. The term "substantially free of other cellular proteins" (also referred to herein as "substantially free of other contaminating proteins") is defined as encompassing individual preparations of each of the component proteins comprising less than 20% (by dry weight) contaminating protein, and preferably comprises less than 5% contaminating protein. Functional forms of each of the component proteins can be prepared as purified preparations by using a cloned gene as described in the attached examples.

By "purified", it is meant, when referring to component protein preparations used to generate a reconstituted protein mixture, that the indicated molecule is present in the substantial absence of other biological macromolecules, such as other proteins (particularly other proteins which may substantially mask, diminish, confuse or alter the characteristics of the component proteins either as purified preparations or in their function in the subject reconstituted mixture). The term "purified" as used herein preferably means at least 80% by dry weight, more preferably in the range of 85% by weight, more preferably 95-99% by weight, and most preferably at least 99.8% by weight, of biological macromolecules of the same type present (but water, buffers, and other small molecules, especially molecules having a molecular weight of less than 5000, can be present). The term "pure" as used herein preferably has the same numerical limits as "purified" immediately above.

A "receptor" or "protein having a receptor function" is a protein that interacts with an extracellular ligand or a ligand that is within the cell but in a space that is topologically equivalent to the extracellular space (eg. inside the Golgi, inside the endoplasmic reticulum, inside the nuclear membrane, inside a lysosome or transport vesicle, etc.). Exemplary receptors are identified herein by annotation as such in various public databases. Receptors often have membrane domains.

A "recombinant nucleic acid" is any nucleic acid that has been placed adjacent to another nucleic acid by recombinant DNA techniques. A "recombined nucleic acid" also includes any nucleic acid that has been placed next to a second nucleic acid by a laboratory genetic technique such as, for example, transformation

and integration, transposon hopping or viral insertion. In general, a recombined nucleic acid is not naturally located adjacent to the second nucleic acid.

The term "recombinant protein" refers to a protein of the present invention which is produced by recombinant DNA techniques, wherein generally DNA
 5 encoding the expressed protein is inserted into a suitable expression vector which is in turn used to transform a host cell to produce the heterologous protein. Moreover, the phrase "derived from", with respect to a recombinant gene encoding the recombinant protein is meant to include within the meaning of "recombinant
 10 amino acid sequence similar thereto which is generated by mutations including substitutions and deletions of a naturally occurring protein.

A "RING domain" or "Ring Finger" is a zinc-binding domain with a defined octet of cysteine and histidine residues. Certain RING domains comprise the consensus sequences as set forth below (amino acid nomenclature is as set forth in
 15 Table 1): Cys Xaa Xaa Cys Xaa₁₀₋₂₀ Cys Xaa His Xaa₂₋₅ Cys Xaa Xaa Cys Xaa₁₃₋₅₀ Cys Xaa Xaa Cys or Cys Xaa Xaa Cys Xaa₁₀₋₂₀ Cys Xaa His Xaa₂₋₅ His Xaa Xaa Cys Xaa₁₃₋₅₀ Cys Xaa Xaa Cys. Certain RING domains are represented as amino acid sequences that are at least 80% identical to amino acids 12-52 of SEQ ID NO: 2 and is set forth in SEQ ID No: 26. Preferred RING domains are 85%, 90%, 95%,
 20 98% and, most preferably, 100% identical to the amino acid sequence of SEQ ID NO: 26. Preferred RING domains of the invention bind to various protein partners to form a complex that has ubiquitin ligase activity. RING domains preferably interact with at least one of the following protein types: F box proteins, E2 ubiquitin conjugating enzymes and cullins.

25 The term "RNA interference" or "RNAi" refers to any method by which expression of a gene or gene product is decreased by introducing into a target cell one or more double-stranded RNAs which are homologous to the gene of interest (particularly to the messenger RNA of the gene of interest).

"Small molecule" as used herein, is meant to refer to a composition, which
 30 has a molecular weight of less than about 5 kD and most preferably less than about 2.5 kD. Small molecules can be nucleic acids, peptides, polypeptides, peptidomimetics, carbohydrates, lipids or other organic (carbon containing) or

inorganic molecules. Many pharmaceutical companies have extensive libraries of chemical and/or biological mixtures comprising arrays of small molecules, often fungal, bacterial, or algal extracts, which can be screened with any of the assays of the invention.

5 An "SH3" or "Src Homology 3" domain is a protein domain of generally about 60 amino acid residues first identified as a conserved sequence in the non-catalytic part of several cytoplasmic protein tyrosine kinases (e.g. Src, Abl, Lck). SH3 domains mediate assembly of specific protein complexes via binding to proline-rich peptides. Exemplary SH3 domains are represented by amino acids 137-
10 192, 199-258, 448-505 and 832-888 of SEQ ID NO:2 and are set forth in SEQ ID Nos: 27-30. In certain embodiments, an SH3 domain interacts with a consensus sequence of RXaaXaaPXaaX6P (where X6, as defined in table 1 below, is a hydrophobic amino acid). In certain embodiments, an SH3 domain interacts with one or more of the following sequences: P(T/S)AP, PFRDY, RPEPTAP,
15 RQGPKEP, RQGPKEPFR, RPEPTAPEE and RPLPVAP.

As used herein, the term "specifically hybridizes" refers to the ability of a nucleic acid probe/primer of the invention to hybridize to at least 12, 15, 20, 25, 30, 35, 40, 45, 50 or 100 consecutive nucleotides of a POSH sequence, or a sequence complementary thereto, or naturally occurring mutants thereof, such that it has less
20 than 15%, preferably less than 10%, and more preferably less than 5% background hybridization to a cellular nucleic acid (e.g., mRNA or genomic DNA) other than the POSH gene. A variety of hybridization conditions may be used to detect specific hybridization, and the stringency is determined primarily by the wash stage of the hybridization assay. Generally high temperatures and low salt concentrations give
25 high stringency, while low temperatures and high salt concentrations give low stringency. Low stringency hybridization is achieved by washing in, for example, about 2.0 x SSC at 50 °C, and high stringency is achieved with about 0.2 x SSC at 50 °C. Further descriptions of stringency are provided below.

As applied to polypeptides, "substantial sequence identity" means that two
30 peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap which share at least 90 percent sequence identity, preferably at least 95 percent sequence identity, more preferably at least 99 percent

sequence identity or more. Preferably, residue positions which are not identical differ by conservative amino acid substitutions. For example, the substitution of amino acids having similar chemical properties such as charge or polarity are not likely to effect the properties of a protein. Examples include glutamine for
5 asparagine or glutamic acid for aspartic acid.

"Transcriptional regulatory sequence" is a generic term used throughout the specification to refer to DNA sequences, such as initiation signals, enhancers, and promoters, which induce or control transcription of protein coding sequences with which they are operably linked. In preferred embodiments, transcription of a
10 recombinant protein gene is under the control of a promoter sequence (or other transcriptional regulatory sequence) which controls the expression of the recombinant gene in a cell-type in which expression is intended. It will also be understood that the recombinant gene can be under the control of transcriptional regulatory sequences which are the same or which are different from those
15 sequences which control transcription of the naturally-occurring form of the protein.

As used herein, a "transgenic animal" is any animal, preferably a non-human mammal, bird or an amphibian, in which one or more of the cells of the animal contain heterologous nucleic acid introduced by way of human intervention, such as by transgenic techniques well known in the art. The nucleic acid is introduced into
20 the cell, directly or indirectly by introduction into a precursor of the cell, by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus. The term genetic manipulation does not include classical cross-breeding, or in vitro fertilization, but rather is directed to the introduction of a recombinant DNA molecule. This molecule may be integrated within a
25 chromosome, or it may be extrachromosomally replicating DNA. In the typical transgenic animals described herein, the transgene causes cells to express a recombinant human POSH protein. The "non-human animals" of the invention include vertebrates such as rodents, non-human primates, sheep, dog, cow, chickens, amphibians, reptiles, etc. Preferred non-human animals are selected from the rodent
30 family including rat and mouse, most preferably mouse, though transgenic amphibians, such as members of the *Xenopus* genus, and transgenic chickens can also provide important tools for understanding and identifying agents which can

affect, for example, embryogenesis and tissue formation. The term "chimeric animal" is used herein to refer to animals in which the recombinant gene is found, or in which the recombinant is expressed in some but not all cells of the animal. The term "tissue specific chimeric animal" indicates that the recombinant human POSH
5 genes is present and/or expressed in some tissues but not others.

As used herein, the term "transgene" means a nucleic acid sequence (encoding, e.g., human POSH polypeptides), which is partly or entirely heterologous, i.e., foreign, to the transgenic animal or cell into which it is introduced, or, is homologous to an endogenous gene of the transgenic animal or cell
10 into which it is introduced, but which is designed to be inserted, or is inserted, into the animal's genome in such a way as to alter the genome of the cell into which it is inserted (e.g., it is inserted at a location which differs from that of the natural gene or its insertion results in a knockout). A transgene can include one or more transcriptional regulatory sequences and any other nucleic acid, such as introns, that
15 may be necessary for optimal expression of a selected nucleic acid.

As is well known, genes for a particular polypeptide may exist in single or multiple copies within the genome of an individual. Such duplicate genes may be identical or may have certain modifications, including nucleotide substitutions, additions or deletions, which all still code for polypeptides having substantially the
20 same activity.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of preferred vector is an episome, i.e., a nucleic acid capable of extra-chromosomal replication. Preferred vectors are those capable of autonomous replication and/expression of
25 nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer to circular double stranded DNA loops which, in their vector form are not bound to the chromosome. In the present
30 specification, "plasmid" and "vector" are used interchangeably as the plasmid is the

most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

5 A "virion" is a complete viral particle; nucleic acid and capsid (and a lipid envelope in some viruses).

*Table 1: Abbreviations for classes of amino acids**

Symbol	Category	Amino Acids Represented
X1	Alcohol	Ser, Thr
X2	Aliphatic	Ile, Leu, Val
Xaa	Any	Ala, Cys, Asp, Glu, Phe, Gly, His, Ile, Lys, Leu, Met, Asn, Pro, Gln, Arg, Ser, Thr, Val, Trp, Tyr
X4	Aromatic	Phe, His, Trp, Tyr
X5	Charged	Asp, Glu, His, Lys, Arg
X6	Hydrophobic	Ala, Cys, Phe, Gly, His, Ile, Lys, Leu, Met, Thr, Val, Trp, Tyr
X7	Negative	Asp, Glu
X8	Polar	Cys, Asp, Glu, His, Lys, Asn, Gln, Arg, Ser, Thr
X9	Positive	His, Lys, Arg
X10	Small	Ala, Cys, Asp, Gly, Asn,

		Pro, Ser, Thr, Val
X11	Tiny	Ala, Gly, Ser
X12	Turnlike	Ala, Cys, Asp, Glu, Gly, His, Lys, Asn, Gln, Arg, Ser, Thr
X13	Asparagine-Aspartate	Asn, Asp

* Abbreviations as adopted from http://smart.embl-heidelberg.de/SMART_DATA/alignments/consensus/grouping.html.

2. Overview

5 In certain aspects, the invention relates to novel human POSH nucleic acids and proteins, and related methods and compositions. In certain aspects, the invention relates to novel associations between certain disease states and POSH nucleic acids and proteins. POSH intersects with and regulates a wide range of key cellular functions that may be manipulated by affecting the level of and/or activity of

10 POSH polypeptides. In certain aspects, by identifying the human POSH gene the present invention provides methods for identifying diseases that are associated with defects in the POSH gene and methods for ameliorating such diseases. Such defects include altered expression level or altered function of POSH. In further aspects, the invention provides nucleic acid agents (e.g. RNAi probes, antisense), antibody-

15 related agents, small molecules and other agents that affect POSH expression level and/or function. In further aspects, the invention provides methods for identifying agents that affect POSH expression level and/or function, and the function of proteins that associate with POSH and/or participate in a POSH mediated process. Other aspects and embodiments are described herein.

20 In certain aspects, the invention relates to the discovery that certain POSH polypeptides function as E3 enzymes in the ubiquitination system. Accordingly, downregulation or upregulation of POSH ubiquitin ligase activity can be used to manipulate biological processes that are affected by protein ubiquitination.

Downregulation or upregulation may be achieved at any stage of POSH formation and regulation, including transcriptional, translational or post-translational regulation. For example, POSH transcript levels may be decreased by RNAi targeted at a POSH gene sequence. As another example, POSH ubiquitin ligase activity may be inhibited by contacting POSH with an antibody that binds to and interferes with a POSH RING domain or a domain of POSH that mediates interaction with a target protein (a protein that is ubiquitinated at least in part because of POSH activity). As another example, POSH activity may be increased by causing increased expression of POSH or an active portion thereof. A ubiquitin ligase, such as POSH, may participate in biological processes such as cellular proliferation and cell death. POSH may participate in diseases characterized by the accumulation of ubiquitinated proteins, such as dementias (e.g. Alzheimer's and Pick's), inclusion body myositis and myopathies, polyglucosan body myopathy, and certain forms of amyotrophic lateral sclerosis. POSH may participate in diseases characterized by the excessive or inappropriate ubiquitination and/or protein degradation. In addition, POSH may participate in oncological processes, such as the failure of cell division control systems, the failure of cell death regulatory systems, and the failure to downregulate hyperactive oncogenes, such as hyperactive membrane-bound growth factor receptors. By identifying certain POSH polypeptides as ubiquitin ligases, aspects of the present invention permit one of ordinary skill in the art to identify diseases that are associated with an altered POSH ubiquitin ligase activity.

In certain aspects, the invention relates to the discovery that hPOSH interacts with Rac, a small GTPase. Rho, Rac and Cdc42 operate together to regulate organization of the actin cytoskeleton and the JNK MAP kinase pathway. Ectopic expression of mouse POSH ("mPOSH") activates the JNK pathway and causes nuclear localization of NF- κ B. Overexpression of mPOSH in fibroblasts stimulates apoptosis. (Tapon et al. (1998) EMBO J. 17:1395-404). In *Drosophila*, POSH may interact, or otherwise influence the signaling of, another GTPase, Ras. (Schnorr et al. (2001) Genetics 159: 609-22). The JNK pathway and NF- κ B regulate a variety of key genes involved in, for example, immune responses, inflammation, cellular proliferation and apoptosis. For example, NF- κ B regulates the production of

interleukin 1, interleukin 8, tumor necrosis factor and many cell adhesion molecules. NF- κ B has both pro-apoptotic and anti-apoptotic roles in the cell (e.g. in FAS-induced cell death and TNF- α signaling, respectively). NF- κ B is negatively regulated, in part, by the inhibitor proteins I κ B α and I κ B β (collectively termed
5 "I κ B"). Phosphorylation of I κ B permits activation and nuclear localization of NF- κ B. Phosphorylation of I κ B triggers its degradation by the ubiquitin system. Accordingly, in yet another embodiment, a POSH polypeptide stimulates the JNK pathway. In an additional embodiment, a POSH polypeptide promotes nuclear localization of NF- κ B. In further embodiments, manipulation of POSH levels
10 and/or activities may be used to manipulate apoptosis.

In further embodiments, POSH participates in cellular proliferation. For example, inhibition of a POSH activity, such as by inhibiting an enzymatic activity or by decreasing cellular levels of POSH polypeptide (e.g., by affecting production or inactivation/destruction of POSH transcripts or POSH polypeptides), decreases
15 proliferation in a cell population. Optionally, inhibition of a POSH activity decreases proliferation in a neoplastic cell, and a neoplastic condition may be treated by inhibiting a POSH activity. A decrease in proliferation may be due to, for example, increased cell death (e.g., apoptosis) or less frequent cell division. In certain embodiments, POSH participates in cellular proliferation by affecting one or
20 more of the signaling pathways described above. In further embodiments, POSH participates in cellular proliferation by participating in the decrease of tumor suppressor activity, for example by participating in a decrease in activity (e.g., by decreasing protein levels, decreasing transcript levels, decreasing protein activity) of a tumor suppressor factor such as p53 or Rb.

25 In a still further embodiment, a POSH polypeptide associates with a vesicular trafficking complex, such as a clathrin- or coatamer- containing complex, and particularly a trafficking complex that localizes to the nucleus and/or Golgi apparatus. Many secreted or transmembrane proteins that pass through the secretory pathway are involved in cellular proliferation. For example, phospholipase D
30 (PLD), is a protein that passes through the secretory pathway, and PLD is associated with certain cancers. PLD mRNA and protein levels are increased in breast cancer

tissues, and PLD alleles are associated with susceptibility to colorectal cancer (Noh, DY et al. (2000) Cancer Lett 161:207-14; Yamada, Y et al. (2003) J. Mol. Med. 81:126-31). Accordingly, inhibition of POSH may be used to inhibit cancers that depend on proteins that are processed by the secretory pathway, such as, for example, PLD.

3. Exemplary Nucleic Acids and Expression Vectors

In certain aspects the invention provides nucleic acids encoding POSH polypeptides, such as, for example, SEQ ID Nos: 2, 5, 7, 9, 11, 26, 27, 28, 29 and 30. Nucleic acids of the invention are further understood to include nucleic acids that comprise variants of SEQ ID Nos:1, 3, 4, 6, 8, 10, 31, 32, 33, 34, and 35. Variant nucleotide sequences include sequences that differ by one or more nucleotide substitutions, additions or deletions, such as allelic variants; and will, therefore, include coding sequences that differ from the nucleotide sequence of the coding sequence designated in SEQ ID Nos:1, 3, 4, 6, 8 10, 31, 32, 33, 34, and 35, e.g., due to the degeneracy of the genetic code. In other embodiments, variants will also include sequences that will hybridize under highly stringent conditions to a nucleotide sequence of a coding sequence designated in any of SEQ ID Nos:1, 3, 4, 6, 8 10, 31, 32, 33, 34, and 35. Preferred nucleic acids of the invention are human POSH sequences, including, for example, any of SEQ ID Nos: 1, 3, 4, 6, 31, 32, 33, 34, 35 and variants thereof and nucleic acids encoding an amino acid sequence selected from among SEQ ID Nos: 2, 5, 7, 26, 27, 28, 29 and 30.

One of ordinary skill in the art will understand readily that appropriate stringency conditions which promote DNA hybridization can be varied. For example, one could perform the hybridization at 6.0 x sodium chloride/sodium citrate (SSC) at about 45 °C, followed by a wash of 2.0 x SSC at 50 °C. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50 °C to a high stringency of about 0.2 x SSC at 50 °C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22 °C, to high stringency conditions at about 65 °C. Both temperature and salt may be varied, or temperature or salt concentration may be held constant while the other variable is changed. In

one embodiment, the invention provides nucleic acids which hybridize under low stringency conditions of 6 x SSC at room temperature followed by a wash at 2 x SSC at room temperature.

Isolated nucleic acids which differ from SEQ ID Nos:1, 3, 4, 6, 8, 10, 31, 32, 33, 34, and 35 due to degeneracy in the genetic code are also within the scope of the invention. For example, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid, or synonyms (for example, CAU and CAC are synonyms for histidine) may result in "silent" mutations which do not affect the amino acid sequence of the protein. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequences of the subject proteins will exist among mammalian cells. One skilled in the art will appreciate that these variations in one or more nucleotides (up to about 3-5% of the nucleotides) of the nucleic acids encoding a particular protein may exist among individuals of a given species due to natural allelic variation. Any and all such nucleotide variations and resulting amino acid polymorphisms are within the scope of this invention.

Optionally, a POSH nucleic acid of the invention will genetically complement a partial or complete POSH loss of function phenotype in a cell. For example, a POSH nucleic acid of the invention may be expressed in a cell in which endogenous POSH has been reduced, possibly by endogenous genetic mutation of the cell or by introduction of RNAi, and the introduced POSH nucleic acid will mitigate a phenotype resulting from the endogenous POSH reduction. An exemplary POSH loss of function phenotype is a decrease in cellular proliferation (as indicated, for example, by a decrease in the rate of cell count increase) of a proliferating cell culture, such as an immortal cell line (e.g. a HeLa cell line). In certain embodiments, a POSH nucleic acid, when expressed at an effective level in a cell, induces apoptosis.

Another aspect of the invention relates to POSH nucleic acids that are used for antisense, RNAi or ribozymes. As used herein, nucleic acid therapy refers to administration or *in situ* generation of a nucleic acid or a derivative thereof which specifically hybridizes (e.g. binds) under cellular conditions with the cellular mRNA and/or genomic DNA encoding one of the subject POSH polypeptides so as to

inhibit production of that protein, e.g. by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix.

5 A nucleic acid therapy construct of the present invention can be delivered, for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the cellular mRNA which encodes a POSH polypeptide. Alternatively, the construct is an oligonucleotide which is generated *ex vivo* and which, when introduced into the cell
10 causes inhibition of expression by hybridizing with the mRNA and/or genomic sequences encoding a POSH polypeptide. Such oligonucleotide probes are optionally modified oligonucleotide which are resistant to endogenous nucleases, e.g. exonucleases and/or endonucleases, and is therefore stable *in vivo*. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate,
15 phosphothioate and methylphosphonate analogs of DNA (see also U.S. Patents 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in nucleic acid therapy have been reviewed, for example, by van der Krol et al., (1988) *Biotechniques* 6:958-976; and Stein et al., (1988) *Cancer Res* 48:2659-2668.

20 Accordingly, the modified oligomers of the invention are useful in therapeutic, diagnostic, and research contexts. In therapeutic applications, the oligomers are utilized in a manner appropriate for nucleic acid therapy in general.

 Optionally, nucleic acid therapy can be used to reduce POSH gene expression either at the RNA transcript level or at the polypeptide level or both. For
25 example, an RNAi against POSH nucleic acid may be introduced into a cell to reduce the expression of the POSH gene. Reduced POSH expression may lead to the inhibition of cellular proliferation. Accordingly, in certain embodiments, nucleic acid inhibitors of POSH are provided as therapeutic agents for neoplastic conditions, including benign tumors and malignant tumors such as cancers. In a
30 preferred embodiment, nucleic acid inhibitors are effective against thyroid carcinoma, liver cancer (hepatocellular cancer), lung cancer, cervical cancer, ovarian cancer, renal cell carcinoma, lymphoma, osteosarcoma, liposarcoma, leukemia,

breast carcinoma, breast adeno-carcinoma, etc. In an additional embodiment, nucleic acid inhibitors are effective against papillomas (e.g. HPV-related papillomas, condylomata acuminatum), cervical squamous intraepithelial lesions, oral cancer, penile cancer, clear cell sarcoma, etc.

5 In addition to use in therapy, the oligomers of the invention may be used as diagnostic reagents to detect the presence or absence of the POSH DNA or RNA sequences to which they specifically bind, such as for determining the level of expression of a gene of the invention or for determining whether a gene of the invention contains a genetic lesion.

10 In another aspect of the invention, the subject nucleic acid is provided in an expression vector comprising a nucleotide sequence encoding a subject POSH polypeptide and operably linked to at least one regulatory sequence. Regulatory sequences are art-recognized and are selected to direct expression of the POSH polypeptide. Accordingly, the term regulatory sequence includes promoters,
15 enhancers and other expression control elements. Exemplary regulatory sequences are described in Goeddel; *Gene Expression Technology: Methods in Enzymology*, Academic Press, San Diego, CA (1990). For instance, any of a wide variety of expression control sequences that control the expression of a DNA sequence when operatively linked to it may be used in these vectors to express DNA sequences
20 encoding a POSH polypeptide. Such useful expression control sequences, include, for example, the early and late promoters of SV40, tet promoter, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage lambda, the control regions for fd
25 coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α -mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that
30 the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the

expression of any other protein encoded by the vector, such as antibiotic markers, should also be considered.

As will be apparent, the subject gene constructs can be used to cause expression of the subject POSH polypeptides in cells propagated in culture, e.g. to produce proteins or polypeptides, including fusion proteins or polypeptides, for purification.

This invention also pertains to a host cell transfected with a recombinant gene including a coding sequence for one or more of the subject POSH polypeptides. The host cell may be any prokaryotic or eukaryotic cell. For example, a polypeptide of the present invention may be expressed in bacterial cells such as *E. coli*, insect cells (e.g., using a baculovirus expression system), yeast, or mammalian cells. Other suitable host cells are known to those skilled in the art.

Accordingly, the present invention further pertains to methods of producing the subject POSH polypeptides. For example, a host cell transfected with an expression vector encoding a POSH polypeptide can be cultured under appropriate conditions to allow expression of the polypeptide to occur. The polypeptide may be secreted and isolated from a mixture of cells and medium containing the polypeptide. Alternatively, the polypeptide may be retained cytoplasmically and the cells harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The polypeptide can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins, including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies specific for particular epitopes of the polypeptide. In a preferred embodiment, the POSH polypeptide is a fusion protein containing a domain which facilitates its purification, such as a POSH-GST fusion protein, POSH-intein fusion protein, POSH-cellulose binding domain fusion protein, POSH-polyhistidine fusion protein etc.

A nucleotide sequence encoding a POSH polypeptide can be used to produce a recombinant form of the protein via microbial or eukaryotic cellular processes. Ligating the polynucleotide sequence into a gene construct, such as an expression

vector, and transforming or transfecting into hosts, either eukaryotic (yeast, avian, insect or mammalian) or prokaryotic (bacterial) cells, are standard procedures.

A recombinant POSH nucleic acid can be produced by ligating the cloned gene, or a portion thereof, into a vector suitable for expression in either prokaryotic
5 cells, eukaryotic cells, or both. Expression vehicles for production of a recombinant POSH polypeptides include plasmids and other vectors. For instance, suitable vectors for the expression of a POSH polypeptide include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic
10 cells, such as *E. coli*.

A number of vectors exist for the expression of recombinant proteins in yeast. For instance, YEP24, YIP5, YEP51, YEP52, pYES2, and YRP17 are cloning and expression vehicles useful in the introduction of genetic constructs into *S. cerevisiae* (see, for example, Broach et al., (1983) in
15 *Experimental Manipulation of Gene Expression*, ed. M. Inouye Academic Press, p. 83, incorporated by reference herein). These vectors can replicate in *E. coli* due the presence of the pBR322 ori, and in *S. cerevisiae* due to the replication determinant of the yeast 2 micron plasmid. In addition, drug resistance markers such as ampicillin can be used.

20 The preferred mammalian expression vectors contain both prokaryotic sequences to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNA1/amp, pcDNA1/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of
25 mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papilloma virus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used
30 for transient expression of proteins in eukaryotic cells. Examples of other viral (including retroviral) expression systems can be found below in the description of gene therapy delivery systems. The various methods employed in the preparation of

the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press, 1989) Chapters 16 and 17. In some instances, it may be desirable to express the recombinant POSH polypeptide by the use of a baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the β -gal containing pBlueBac III).

It is well known in the art that a methionine at the N-terminal position can be enzymatically cleaved by the use of the enzyme methionine aminopeptidase (MAP). MAP has been cloned from *E. coli* (Ben-Bassat et al., (1987) *J. Bacteriol.* 169:751-757) and *Salmonella typhimurium* and its *in vitro* activity has been demonstrated on recombinant proteins (Miller et al., (1987) *PNAS USA* 84:2718-1722). Therefore, removal of an N-terminal methionine, if desired, can be achieved either *in vivo* by expressing such recombinant polypeptides in a host which produces MAP (e.g., *E. coli* or CM89 or *S. cerevisiae*), or *in vitro* by use of purified MAP (e.g., procedure of Miller et al.).

Alternatively, the coding sequences for the polypeptide can be incorporated as a part of a fusion gene including a nucleotide sequence encoding a different polypeptide. This type of expression system can be useful under conditions where it is desirable, e.g., to produce an immunogenic fragment of a POSH polypeptide. For example, the VP6 capsid protein of rotavirus can be used as an immunologic carrier protein for portions of polypeptide, either in the monomeric form or in the form of a viral particle. The nucleic acid sequences corresponding to the portion of the POSH polypeptide to which antibodies are to be raised can be incorporated into a fusion gene construct which includes coding sequences for a late vaccinia virus structural protein to produce a set of recombinant viruses expressing fusion proteins comprising a portion of the protein as part of the virion. The Hepatitis B surface antigen can also be utilized in this role as well. Similarly, chimeric constructs coding for fusion proteins containing a portion of a POSH polypeptide and the

poliovirus capsid protein can be created to enhance immunogenicity (see, for example, EP Publication NO: 0259149; and Evans et al., (1989) *Nature* 339:385; Huang et al., (1988) *J. Virol.* 62:3855; and Schlienger et al., (1992) *J. Virol.* 66:2).

The Multiple Antigen Peptide system for peptide-based immunization can be
 5 utilized, wherein a desired portion of a POSH polypeptide is obtained directly from organo-chemical synthesis of the peptide onto an oligomeric branching lysine core (see, for example, Posnett et al., (1988) *JBC* 263:1719 and Nardelli et al., (1992) *J. Immunol.* 148:914). Antigenic determinants of a POSH polypeptide can also be expressed and presented by bacterial cells.

10 In another embodiment, a fusion gene coding for a purification leader sequence, such as a poly-(His)/enterokinase cleavage site sequence at the N-terminus of the desired portion of the recombinant protein, can allow purification of the expressed fusion protein by affinity chromatography using a Ni²⁺ metal resin. The purification leader sequence can then be subsequently removed by treatment
 15 with enterokinase to provide the purified POSH polypeptide (e.g., see Hochuli et al., (1987) *J. Chromatography* 411:177; and Janknecht et al., *PNAS USA* 88:8972).

Techniques for making fusion genes are well known. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-
 20 ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be
 25 carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al., John Wiley & Sons: 1992).

30 Table 2: Exemplary POSH nucleic acids

<u>Sequence Name</u>	<u>Organism</u>	<u>Accession Number</u>
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cDNA FLJ11367 fis, clone HEMBA1000303	Homo sapiens	AK021429
Plenty of SH3 domains (POSH) mRNA	Mus musculus	NM_021506
Plenty of SH3s (POSH) mRNA	Mus musculus	AF030131
Plenty of SH3s (POSH) mRNA	Drosophila melanogaster	NM_079052
Plenty of SH3s (POSH) mRNA	Drosophila melanogaster	AF220364

Table 3: Exemplary POSH polypeptides

<u>Sequence Name</u>	<u>Organism</u>	<u>Accession Number</u>
SH3 domains-containing protein POSH	Mus musculus	T09071
plenty of SH3 domains	Mus musculus	NP_067481
Plenty of SH3s; POSH	Mus musculus	AAC40070
Plenty of SH3s	Drosophila melanogaster	AAF37265
LD45365p	Drosophila melanogaster	AAK93408
POSH gene product	Drosophila melanogaster	AAF57833
Plenty of SH3s	Drosophila melanogaster	NP_523776

In addition the following Tables provide the nucleic acid sequence and related SEQ
5 ID NOs for domains of human POSH protein and a summary of sequence identification numbers used in this application.

Table 4. Nucleic Acid Sequences and related SEQ ID NOs for domains in human POSH

Name of the sequence	Sequence	SEQ ID NO.
RING domain	TGTCCGGTGTGTCTAGAGCGCCTTGATGCTTCTGCGAAGGTCT TGCCTTGCCAGCATACGTTTTGCAAGCGATGTTTGCT GGGGATCGTAGGTTCTCGAAATGAACTCAGATGTCCCGAGT	31
1 st SH ₃ domain	CCATGTGCCAAAGCGTTATACAACATGAAGGAAAAGAGCCTG GAGACCTTAAATTCAGCAAAGGCGACATCATCATTTT GCGAAGACAAGTGGATGAAAATTGGTACCATGGGGAAGTCAAT GGAATCCATGGCTTTTTCCCACTTTGTGCAGA TTATT	32
2 nd SH ₃ domain	CCTCAGTGCAAAGCACTTTATGACTTTGAAGTGAAAGACAAGG AAGCAGACAAAGATTGCCTTCCATTTGCAAAGGATGA TGTCTGACTGTGATCCGAAGAGTGGATGAAAAGTGGGCTGAA GGAATGCTGGCAGACAAAATAGGAATATTTCCAATTT CATATGTTGAGTTTAAC	33
3 rd SH ₃ domain	AGTGTGTATGTTGCTATATATCCATACACTCCTCGGAAAGAGG ATGAACTAGAGCTGAGAAAAGGGGAGATGTTTTTAGT GTTTGAGCGCTGCCAGGATGGCTGGTTCAAAGGGACATCCATG CATACCAGCAAGATAGGGGTTTTCCCTGGCAATTATG TGGCACCAGTC	34
4 th SH ₃ domain	GAAAGGCACAGGGTGGTGGTTTCCTATCCTCCTCAGAGTGAGG CAGAACTTGAACCTAAAGAAGGAGATATTGTGTTTGT TCATAAAAAACGAGAGGATGGCTGGTTCAAAGGCACATTACAA CGTAATGGGAAAAGTGGCCTTTTCCCAGGAAGCTTTG TGGAAAACA	35

Table 5. Summary of Sequence Identification Numbers

Sequence Information	Sequence Identification Number (SEQ ID NO)
Human POSH Coding Sequence	SEQ ID No: 1
Human POSH Amino Acid Sequence	SEQ ID No: 2
Human POSH cDNA Sequence	SEQ ID No: 3
5' cDNA Fragment of Human POSH	SEQ ID No: 4
N-terminus Protein Fragment of Human POSH	SEQ ID No: 5
3' mRNA Fragment of Human POSH	SEQ ID No: 6
C-terminus Protein Fragment of Human POSH	SEQ ID No: 7
Mouse POSH mRNA Sequence	SEQ ID No: 8
Mouse POSH Protein Sequence	SEQ ID No: 9
Drosophila melanogaster POSH mRNA Sequence	SEQ ID No: 10
Drosophila melanogaster POSH Protein Sequence	SEQ ID No: 11
Human POSH RING Domain Amino Acid Sequence	SEQ ID No: 26
Human POSH 1 st SH ₃ Domain Amino Acid Sequence	SEQ ID No: 27
Human POSH 2 nd SH ₃ Domain Amino Acid Sequence	SEQ ID No: 28
Human POSH 3 rd SH ₃ Domain Amino Acid Sequence	SEQ ID No: 29
Human POSH 4 th SH ₃ Domain Amino Acid Sequence	SEQ ID No: 30
Human POSH RING Domain Nucleic Acid Sequence	SEQ ID No: 31
Human POSH 1 st SH ₃ Domain Nucleic Acid Sequence	SEQ ID No: 32
Human POSH 2 nd SH ₃ Domain Nucleic Acid Sequence	SEQ ID No: 33
Human POSH 3 rd SH ₃ Domain Nucleic Acid Sequence	SEQ ID No: 34
Human POSH 4 th SH ₃ Domain Nucleic Acid Sequence	SEQ ID No: 35

4. Exemplary Polypeptides

The present invention also makes available isolated and/or purified forms of the subject POSH polypeptides, which are isolated from, or otherwise substantially
5 free of, other intracellular proteins which might normally be associated with the protein or a particular complex including the protein. In certain embodiments, POSH polypeptides have an amino acid sequence that is at least 60% identical to an amino acid sequence as set forth in any of SEQ ID Nos: 2, 5, 7, 9, 11, 26, 27, 28, 29
10 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to an amino acid sequence as set forth in any of SEQ ID Nos: 2, 5, 7, 9, 11, 26, 27, 28, 29 and 30.

Optionally, a POSH polypeptide of the invention will function in place of an endogenous POSH polypeptide, for example by mitigating a partial or complete
15 POSH loss of function phenotype in a cell. For example, a POSH polypeptide of the invention may be produced in a cell in which endogenous POSH has been reduced, possibly by endogenous genetic mutation of the cell or by introduction of RNAi, and the introduced POSH polypeptide will mitigate a phenotype resulting from the endogenous POSH reduction. A further exemplary POSH loss of function
20 phenotype is a decrease in cellular proliferation (as indicated, for example, by a decrease in the rate of cell count increase) of a proliferating cell culture, such as an immortal cell line (e.g. a HeLa cell line). In certain embodiments, a POSH polypeptide, when produced at an effective level in a cell, induces apoptosis.

In certain embodiments, a POSH polypeptide of the invention interacts with
25 a viral Gag protein through one or more SH3 domain. In additional embodiments, POSH polypeptides may also, or alternatively, function in ubiquitylation in part through the activity of a RING domain.

In another aspect, the invention provides polypeptides that are agonists or antagonists of a POSH polypeptide. Variants and fragments of a POSH polypeptide
30 may have a hyperactive or constitutive activity, or, alternatively, act to prevent POSH polypeptides from performing one or more functions. For example, a truncated form lacking one or more domain may have a dominant negative effect.

Another aspect of the invention relates to polypeptides derived from a full-length POSH polypeptide. Isolated peptidyl portions of the subject proteins can be obtained by screening polypeptides recombinantly produced from the corresponding fragment of the nucleic acid encoding such polypeptides. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, any one of the subject proteins can be arbitrarily divided into fragments of desired length with no overlap of the fragments, or preferably divided into overlapping fragments of a desired length. The fragments can be produced (recombinantly or by chemical synthesis) and tested to identify those peptidyl fragments which can function as either agonists or antagonists of the formation of a specific protein complex, or more generally of a POSH complex, such as by microinjection assays.

It is also possible to modify the structure of the subject POSH polypeptides for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., ex vivo shelf life and resistance to proteolytic degradation in vivo). Such modified polypeptides, when designed to retain at least one activity of the naturally-occurring form of the protein, are considered functional equivalents of the POSH polypeptides described in more detail herein. Such modified polypeptides can be produced, for instance, by amino acid substitution, deletion, or addition.

For instance, it is reasonable to expect, for example, that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid (i.e. conservative mutations) will not have a major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids can be divided into four families: (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine; (3) nonpolar = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar = glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In similar fashion, the amino acid repertoire can be grouped as (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine histidine,

(3) aliphatic = glycine, alanine, valine, leucine, isoleucine, serine, threonine, with serine and threonine optionally be grouped separately as aliphatic-hydroxyl; (4) aromatic = phenylalanine, tyrosine, tryptophan; (5) amide = asparagine, glutamine; and (6) sulfur -containing = cysteine and methionine. (see, for example, 5 Biochemistry, 2nd ed., Ed. by L. Stryer, W.H. Freeman and Co., 1981). Whether a change in the amino acid sequence of a polypeptide results in a functional homolog can be readily determined by assessing the ability of the variant polypeptide to produce a response in cells in a fashion similar to the wild-type protein. For instance, such variant forms of a POSH polypeptide can be assessed, e.g., for their 10 ability to bind to another polypeptide, e.g., another POSH polypeptide or another protein involved in cellular proliferation. Polypeptides in which more than one replacement has taken place can readily be tested in the same manner.

This invention further contemplates a method of generating sets of combinatorial mutants of the subject POSH polypeptides, as well as truncation 15 mutants, and is especially useful for identifying potential variant sequences (e.g. homologs) that are functional in binding to a POSH polypeptide. The purpose of screening such combinatorial libraries is to generate, for example, POSH homologs which can act as either agonists or antagonist, or alternatively, which possess novel activities all together. Combinatorially-derived homologs can be generated which 20 have a selective potency relative to a naturally occurring POSH polypeptide. Such proteins, when expressed from recombinant DNA constructs, can be used in gene therapy protocols.

Likewise, mutagenesis can give rise to homologs which have intracellular half-lives dramatically different than the corresponding wild-type protein. For 25 example, the altered protein can be rendered either more stable or less stable to proteolytic degradation or other cellular process which result in destruction of, or otherwise inactivation of the POSH polypeptide of interest. Such homologs, and the genes which encode them, can be utilized to alter POSH levels by modulating the half-life of the protein. For instance, a short half-life can give rise to more transient 30 biological effects and, when part of an inducible expression system, can allow tighter control of recombinant POSH levels within the cell. As above, such proteins,

and particularly their recombinant nucleic acid constructs, can be used in gene therapy protocols.

In similar fashion, POSH homologs can be generated by the present combinatorial approach to act as antagonists, in that they are able to interfere with the ability of the corresponding wild-type protein to function.

In a representative embodiment of this method, the amino acid sequences for a population of POSH homologs are aligned, preferably to promote the highest homology possible. Such a population of variants can include, for example, homologs from one or more species, or homologs from the same species but which differ due to mutation. Amino acids which appear at each position of the aligned sequences are selected to create a degenerate set of combinatorial sequences. In a preferred embodiment, the combinatorial library is produced by way of a degenerate library of genes encoding a library of polypeptides which each include at least a portion of potential POSH sequences. For instance, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential POSH nucleotide sequences are expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g. for phage display).

There are many ways by which the library of potential homologs can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes then be ligated into an appropriate gene for expression. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential POSH sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang, SA (1983) Tetrahedron 39:3; Itakura et al., (1981) Recombinant DNA, Proc. 3rd Cleveland Sympos. Macromolecules, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al., (1984) Annu. Rev. Biochem. 53:323; Itakura et al., (1984) Science 198:1056; Ike et al., (1983) Nucleic Acid Res. 11:477). Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al., (1990) Science 249:386-390; Roberts et al., (1992) PNAS USA 89:2429-2433; Devlin et al., (1990) Science 249: 404-406; Cwirla et al., (1990)

PNAS USA 87: 6378-6382; as well as U.S. Patent Nos: 5,223,409, 5,198,346, and 5,096,815).

Alternatively, other forms of mutagenesis can be utilized to generate a combinatorial library. For example, POSH homologs (both agonist and antagonist
5 forms) can be generated and isolated from a library by screening using, for example, alanine scanning mutagenesis and the like (Ruf et al., (1994) *Biochemistry* 33:1565-1572; Wang et al., (1994) *J. Biol. Chem.* 269:3095-3099; Balint et al., (1993) *Gene* 137:109-118; Grodberg et al., (1993) *Eur. J. Biochem.* 218:597-601; Nagashima et al., (1993) *J. Biol. Chem.* 268:2888-2892; Lowman et al., (1991) *Biochemistry*
10 30:10832-10838; and Cunningham et al., (1989) *Science* 244:1081-1085), by linker scanning mutagenesis (Gustin et al., (1993) *Virology* 193:653-660; Brown et al., (1992) *Mol. Cell Biol.* 12:2644-2652; McKnight et al., (1982) *Science* 232:316); by saturation mutagenesis (Meyers et al., (1986) *Science* 232:613); by PCR mutagenesis (Leung et al., (1989) *Method Cell Mol Biol* 1:11-19); or by random
15 mutagenesis, including chemical mutagenesis, etc. (Miller et al., (1992) *A Short Course in Bacterial Genetics*, CSHL Press, Cold Spring Harbor, NY; and Greener et al., (1994) *Strategies in Mol Biol* 7:32-34). Linker scanning mutagenesis, particularly in a combinatorial setting, is an attractive method for identifying truncated (bioactive) forms of POSH polypeptides.

20 A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations and truncations, and, for that matter, for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of POSH homologs. The most widely
25 used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of the illustrative
30 assays described below are amenable to high through-put analysis as necessary to screen large numbers of degenerate sequences created by combinatorial mutagenesis techniques.

In an illustrative embodiment of a screening assay, candidate combinatorial gene products of one of the subject proteins are displayed on the surface of a cell or virus, and the ability of particular cells or viral particles to bind a POSH polypeptide is detected in a "panning assay". For instance, a library of POSH variants can be
5 cloned into the gene for a surface membrane protein of a bacterial cell (Ladner et al., WO 88/06630; Fuchs et al., (1991) *Bio/Technology* 9:1370-1371; and Goward et al., (1992) *TIBS* 18:136-140), and the resulting fusion protein detected by panning, e.g. using a fluorescently labeled molecule which binds the POSH polypeptide, to score for potentially functional homologs. Cells can be visually
10 inspected and separated under a fluorescence microscope, or, where the morphology of the cell permits, separated by a fluorescence-activated cell sorter.

In similar fashion, the gene library can be expressed as a fusion protein on the surface of a viral particle. For instance, in the filamentous phage system, foreign peptide sequences can be expressed on the surface of infectious phage, thereby
15 conferring two significant benefits. First, since these phage can be applied to affinity matrices at very high concentrations, a large number of phage can be screened at one time. Second, since each infectious phage displays the combinatorial gene product on its surface, if a particular phage is recovered from an affinity matrix in low yield, the phage can be amplified by another round of
20 infection. The group of almost identical *E. coli* filamentous phages M13, fd, and f1 are most often used in phage display libraries, as either of the phage gIII or gVIII coat proteins can be used to generate fusion proteins without disrupting the ultimate packaging of the viral particle (Ladner et al., PCT publication WO 90/02909; Garrard et al., PCT publication WO 92/09690; Marks et al., (1992) *J. Biol. Chem.*
25 267:16007-16010; Griffiths et al., (1993) *EMBO J.* 12:725-734; Clackson et al., (1991) *Nature* 352:624-628; and Barbas et al., (1992) *PNAS USA* 89:4457-4461).

The invention also provides for reduction of the subject POSH polypeptides to generate mimetics, e.g. peptide or non-peptide agents, which are able to mimic binding of the authentic protein to another cellular partner. Such mutagenic
30 techniques as described above, as well as the thioredoxin system, are also particularly useful for mapping the determinants of a POSH polypeptide which participate in protein-protein interactions involved in, for example, binding of

proteins involved in cellular proliferation to each other. To illustrate, the critical residues of a POSH polypeptide which are involved in molecular recognition of a substrate protein can be determined and used to generate POSH polypeptide-derived peptidomimetics which bind to the substrate protein, and by inhibiting POSH binding, act to inhibit its biological activity. By employing, for example, scanning mutagenesis to map the amino acid residues of a POSH polypeptide which are involved in binding to another polypeptide, peptidomimetic compounds can be generated which mimic those residues involved in binding. For instance, non-hydrolyzable peptide analogs of such residues can be generated using benzodiazepine (e.g., see Freidinger et al., in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al., in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings (Garvey et al., in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al., (1986) J. Med. Chem. 29:295; and Ewenson et al., in Peptides: Structure and Function (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), b-turn dipeptide cores (Nagai et al., (1985) Tetrahedron Lett 26:647; and Sato et al., (1986) J Chem Soc Perkin Trans 1:1231), and b-aminoalcohols (Gordon et al., (1985) Biochem Biophys Res Commun 126:419; and Dann et al., (1986) Biochem Biophys Res Commun 134:71).

The following table provides the sequences of the RING domain and the various SH3 domains.

Table 6. Amino Acid Sequences and related SEQ ID NOs for domains in human

25 POSH

Name of the sequence	Sequence	SEQ ID NO.
RING domain	CPVCLERLDASAKVLPCQHTFCKRCLLGIVGSRNELRCPEC	26
1 st SH ₃ domain	PCAKALYNYEGKEPGDLKFSKGDIIILRRQVDENWYHGEVNGIHGF FPTNFVQIIK	27

2 nd SH ₃ domain	PQCKALYDFEVKDKEADKDCLPFAKDDVLTVIRRVNENWAEGLAD KIGIFPIISYVEFNS	28
3 rd SH ₃ domain	SVYVAIYPYTPRKEDELELRKGEMFLVFERCQDGWFKGTSMHTSKI GVFPGNYVAPVT	29
4 th SH ₃ domain	ERHRVVVSYPQSEAELELKEGDIVFVHKKREDGWFKGTLQRNGKT GLFPGSFVENI	30

5. Antibodies and Uses Thereof

Another aspect of the invention pertains to an antibody specifically reactive with a POSH polypeptide. For example, by using immunogens derived from a POSH polypeptide, e.g., based on the cDNA sequences, anti-protein/anti-peptide antisera or monoclonal antibodies can be made by standard protocols (See, for example, Antibodies: A Laboratory Manual ed. by Harlow and Lane (Cold Spring Harbor Press: 1988)). A mammal, such as a mouse, a hamster or rabbit can be immunized with an immunogenic form of the peptide (e.g., a POSH polypeptide or an antigenic fragment which is capable of eliciting an antibody response, or a fusion protein as described above). Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers or other techniques well known in the art. An immunogenic portion of a POSH polypeptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassays can be used with the immunogen as antigen to assess the levels of antibodies. In a preferred embodiment, the subject antibodies are immunospecific for antigenic determinants of a POSH polypeptide of a mammal, e.g., antigenic determinants of a protein set forth in SEQ ID NO:2.

In one embodiment, antibodies are specific for a RING domain or an SH3 domain, and preferably the domain is part of a POSH polypeptide. In a more specific embodiment, the domain is part of an amino acid sequence set forth in SEQ ID NO:2. In a set of exemplary embodiments, an antibody binds to one or more SH3 domains represented by amino acids 137-192 of SEQ ID NO:2, amino acids 199-258 of SEQ ID NO:2, amino acids 448-505 of SEQ ID NO:2, and/or amino

acids 832-888 of SEQ ID NO:2. In another exemplary embodiment, an antibody binds to a RING domain represented by amino acids 12-52 of SEQ ID NO:2. In another embodiment, the antibodies are immunoreactive with one or more proteins having an amino acid sequence that is at least 80% identical to an amino acid sequence as set forth in SEQ ID NO:2. In other embodiments, an antibody is immunoreactive with one or more proteins having an amino acid sequence that is 85%, 90%, 95%, 98%, 99% or identical to an amino acid sequence as set forth in SEQ ID NO:2.

Following immunization of an animal with an antigenic preparation of a POSH polypeptide, anti-POSH antisera can be obtained and, if desired, polyclonal anti-POSH antibodies isolated from the serum. To produce monoclonal antibodies, antibody-producing cells (lymphocytes) can be harvested from an immunized animal and fused by standard somatic cell fusion procedures with immortalizing cells such as myeloma cells to yield hybridoma cells. Such techniques are well known in the art, and include, for example, the hybridoma technique (originally developed by Kohler and Milstein, (1975) *Nature*, 256: 495-497), the human B cell hybridoma technique (Kozbar et al., (1983) *Immunology Today*, 4: 72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., (1985) *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc. pp. 77-96). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with a mammalian POSH polypeptide of the present invention and monoclonal antibodies isolated from a culture comprising such hybridoma cells. In one embodiment anti-human POSH antibodies specifically react with the protein encoded by a nucleic acid having SEQ ID NO:2.

The term antibody as used herein is intended to include fragments thereof which are also specifically reactive with one of the subject POSH polypeptides. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, F(ab)₂ fragments can be generated by treating antibody with pepsin. The resulting F(ab)₂ fragment can be treated to reduce disulfide bridges to produce Fab fragments. The antibody of the present invention is further intended to include bispecific, single-chain, and chimeric and humanized molecules having affinity for a

POSH polypeptide conferred by at least one CDR region of the antibody. In preferred embodiments, the antibodies, the antibody further comprises a label attached thereto and able to be detected, (e.g., the label can be a radioisotope, fluorescent compound, enzyme or enzyme co-factor).

5 Anti-POSH antibodies can be used, e.g., to monitor POSH polypeptide levels in a patient, particularly for determining whether or not said patient has a neoplastic condition, including cancer, or allowing determination of the efficacy of a given treatment regimen for a patient afflicted with such a neoplastic condition. The level of POSH polypeptide may be measured in a variety of sample types such as, for
10 example, cells and/or in bodily fluid, such as in blood samples or tumor biopsies.

 Another application of anti-POSH antibodies of the present invention is in the immunological screening of cDNA libraries constructed in expression vectors such as gt11, gt18-23, ZAP, and ORF8. Messenger libraries of this type, having coding sequences inserted in the correct reading frame and orientation, can produce
15 fusion proteins. For instance, gt11 will produce fusion proteins whose amino termini consist of β -galactosidase amino acid sequences and whose carboxy termini consist of a foreign polypeptide. Antigenic epitopes of a POSH polypeptide, e.g., other orthologs of a particular protein or other paralogs from the same species, can then be detected with antibodies, as, for example, reacting nitrocellulose filters lifted
20 from infected plates with the appropriate anti-POSH antibodies. Positive phage detected by this assay can then be isolated from the infected plate. Thus, the presence of POSH homologs can be detected and cloned from other animals, as can alternate isoforms (including splice variants) from humans.

25

6. Homology Searching of Nucleotide and Polypeptide Sequences

 The nucleotide or amino acid sequences of the invention may be used as query sequences against databases such as GenBank, SwissProt, BLOCKS, and
30 Pima II. These databases contain previously identified and annotated sequences that can be searched for regions of homology (similarity) using BLAST, which stands for

Basic Local Alignment Search Tool (Altschul S F (1993) J Mol Evol 36:290-300; Altschul, S F et al (1990) J Mol Biol 215:403-10).

BLAST produces alignments of both nucleotide and amino acid sequences to determine sequence similarity. Because of the local nature of the alignments, BLAST is especially useful in determining exact matches or in identifying homologs which may be of prokaryotic (bacterial) or eukaryotic (animal, fungal or plant) origin. Other algorithms such as the one described in Smith, R. F. and T. F. Smith (1992; Protein Engineering 5:35-51), incorporated herein by reference, can be used when dealing with primary sequence patterns and secondary structure gap penalties. As disclosed in this application, sequences have lengths of at least 49 nucleotides and no more than 12% uncalled bases (where N is recorded rather than A, C, G, or T).

The BLAST approach, as detailed in Karlin and Altschul (1993; Proc Nat Acad Sci 90:5873-7) and incorporated herein by reference, searches matches between a query sequence and a database sequence, to evaluate the statistical significance of any matches found, and to report only those matches which satisfy the user-selected threshold of significance. Preferably the threshold is set at 10-25 for nucleotides and 3-15 for peptides.

7. Transgenic Animals and Uses Thereof

Another aspect of the invention features transgenic non-human animals which express a heterologous POSH gene, preferentially a human POSH gene of the present invention, and/or which have had one or both copies of the endogenous POSH genes disrupted in at least one of the tissue or cell-types of the animal. Accordingly, the invention features an animal model for neoplastic conditions, including cancers. In one embodiment, the transgenic non-human animals is a mammal such as a mouse, rat, rabbit, goat, sheep, dog, cat, cow, or non-human primate. Without being bound to theory, given the role of POSH in cellular proliferation, it is proposed that such an animal may tend to develop neoplastic conditions in which cells lose the normal growth-limitation and over-proliferate. Accordingly, such a transgenic animal may serve as a useful animal model to study the progression of neoplastic conditions, in particular, cancers. Alternatively, such

an animal can be useful as a basis to introduce one or more other human transgenes, to create a transgenic animal carrying multiple human genes involved in cellular proliferation. As a result of the introduction of multiple human transgenes, the transgenic animal may tend to develop neoplastic conditions, and therefore provide
5 an useful animal model to study neoplastic condition, in particular cancers.

Another aspect of the present invention concerns transgenic animals which are comprised of cells (of that animal) which contain a transgene of the present invention and which preferably (though optionally) express an exogenous POSH protein in one or more cells in the animal. A POSH transgene can encode the wild-
10 type form of the protein, or can encode homologs thereof, as well as antisense constructs. Moreover, it may be desirable to express the heterologous POSH transgene conditionally such that either the timing or the level of POSH gene expression can be regulated. Such conditional expression can be provided using prokaryotic promoter sequences which require prokaryotic proteins to be
15 simultaneous expressed in order to facilitate expression of the POSH transgene. Exemplary promoters and the corresponding trans-activating prokaryotic proteins are given in U.S. Pat. No. 4,833,080.

Moreover, transgenic animals exhibiting tissue specific expression can be generated, for example, by inserting a tissue specific regulatory element, such as an
20 enhancer, into the transgene. For example, the endogenous POSH gene promoter or a portion thereof can be replaced with another promoter and/or enhancer, e.g., a CMV or a Moloney murine leukemia virus (MLV) promoter and/or enhancer.

Alternatively, non-human transgenic animals that only express POSH transgenes in the brain can be generated using brain specific promoters (e.g. myelin
25 basic protein (MBP) promoter, the neurofilament protein (NF-L) promoter, the gonadotropin-releasing hormone promoter, the vasopressin promoter and the neuron-specific enolase promoter, see So Forss-Petter et al., Neuron, 5, 187, (1990). Such animals can provide a useful in vivo model to evaluate the ability of a potential anti-HIV drug to cross the blood-brain barrier. Other target cells for which specific
30 promoters can be used are, for example, macrophages, T cells and B cells. Other

tissue specific promoters are well-known in the art, see e.g. R.Jaenisch, Science, 240, 1468 (1988).

Non-human transgenic animals containing an inducible POSH transgene can be generated using inducible regulatory elements (e.g. metallothionein promoter), which are well-known in the art. POSH transgene expression can then be initiated in these animals by administering to the animal a compound which induces gene expression (e.g. heavy metals). Another preferred inducible system comprises a tetracycline-inducible transcriptional activator (U.S. Pat. No. 5,654,168 issued Aug. 5, 1997 to Bujard and Gossen and U.S. Pat. No. 5,650,298 issued Jul. 22, 1997 to Bujard et al.).

In general, transgenic animal lines can be obtained by generating transgenic animals having incorporated into their genome at least one transgene, selecting at least one founder from these animals and breeding the founder or founders to establish at least one line of transgenic animals having the selected transgene incorporated into their genome.

Animals for obtaining eggs or other nucleated cells (e.g. embryonic stem cells) for generating transgenic animals can be obtained from standard commercial sources such as Charles River Laboratories (Wilmington, Mass.), Taconic (Germantown, N.Y.), Harlan Sprague Dawley (Indianapolis, Ind.).

Eggs can be obtained from suitable animals, e.g., by flushing from the oviduct or using techniques described in U.S. Pat. No. 5,489,742 issued Feb. 6, 1996 to Hammer and Taurog; U.S. Pat. No. 5,625,125 issued on Apr. 29, 1997 to Bennett et al.; Gordon et al., 1980, Proc. Natl. Acad. Sci. USA 77:7380-7384; Gordon & Ruddle, 1981, Science 214: 1244-1246; U.S. Pat. No. 4,873,191 to T. E. Wagner and P. C. Hoppe; U.S. Pat. No. 5,604,131; Armstrong, et al. (1988) J. of Reproduction, 39:511 or PCT application No. PCT/FR93/00598 (WO 94/00568) by Mehtali et al. Preferably, the female is subjected to hormonal conditions effective to promote superovulation prior to obtaining the eggs.

Many techniques can be used to introduce DNA into an egg or other nucleated cell, including in vitro fertilization using sperm as a carrier of exogenous DNA ("sperm-mediated gene transfer", e.g., Lavitrano et al., 1989, Cell 57: 717-723), microinjection, gene targeting (Thompson et al., 1989, Cell 56: 313-321),
5 electroporation (Lo, 1983, Mol. Cell. Biol. 3: 1803-1814), transfection, or retrovirus mediated gene transfer (Van der Putten et al., 1985, Proc. Natl. Acad. Sci. USA 82: 6148-6152). For a review of such techniques, see Gordon (1989), Transgenic Animals, Intl. Rev. Cytol. 115:171-229.

Except for sperm-mediated gene transfer, eggs should be fertilized in
10 conjunction with (before, during or after) other transgene transfer techniques. A preferred method for fertilizing eggs is by breeding the female with a fertile male. However, eggs can also be fertilized by in vitro fertilization techniques.

Fertilized, transgene containing eggs can then be transferred to pseudopregnant animals, also termed "foster mother animals", using suitable
15 techniques. Pseudopregnant animals can be obtained, for example, by placing 40-80 day old female animals, which are more than 8 weeks of age, in cages with infertile males, e.g., vasectomized males. The next morning females are checked for vaginal plugs. Females who have mated with vasectomized males are held aside until the time of transfer.

20 Recipient females can be synchronized, e.g. using GNRH agonist (GnRH-a): des-gly10, (D-Ala6)-LH-RH Ethylamide, SigmaChemical Co., St. Louis, Mo. Alternatively, a unilateral pregnancy can be achieved by a brief surgical procedure involving the "peeling" away of the bursa membrane on the left uterine horn. Injected embryos can then be transferred to the left uterine horn via the
25 infundibulum. Potential transgenic founders can typically be identified immediately at birth from the endogenous litter mates. For generating transgenic animals from embryonic stem cells, see e.g. Teratocarcinomas and embryonic stem cells, a practical approach, ed. E. J. Robertson, (IRL Press 1987) or in Potter et al Proc. Natl. Acad. Sci. USA 81, 7161 (1984), the teachings of which are incorporated
30 herein by reference.

Founders that express the gene can then be bred to establish a transgenic line. Accordingly, founder animals can be bred, inbred, crossbred or outbred to produce colonies of animals of the present invention. Animals comprising multiple transgenes can be generated by crossing different founder animals (e.g. an HIV
5 transgenic animal and a transgenic animal, which expresses human CD4), as well as by introducing multiple transgenes into an egg or embryonic cell as described above. Furthermore, embryos from A-transgenic animals can be stored as frozen embryos, which are thawed and implanted into pseudo-pregnant animals when needed (See e.g. Hirabayashi et al. (1997) Exp Anim 46: 111 and Anzai (1994) Jikken Dobutsu
10 43: 247).

The present invention provides for transgenic animals that carry the transgene in all their cells, as well as animals that carry the transgene in some, but not all cells, i.e., mosaic animals. The transgene can be integrated as a single transgene or in tandem, e.g., head to head tandems, or head to tail or tail to tail or as
15 multiple copies.

The successful expression of the transgene can be detected by any of several means well known to those skilled in the art. Non-limiting examples include Northern blot, in situ hybridization of mRNA analysis, Western blot analysis, immunohistochemistry, and FACS analysis of protein expression.

20 In a further aspect, the invention features non-human animal cells containing a POSH transgene, preferentially a human POSH transgene. For example, the animal cell (e.g. somatic cell or germ cell (i.e. egg or sperm)) can be obtained from the transgenic animal. Transgenic somatic cells or cell lines can be used, for example, in drug screening assays. Transgenic germ cells, on the other hand, can be used in
25 generating transgenic progeny, as described above.

The invention further provides methods for identifying (screening) or for determining the safety and/or efficacy of anti-proliferative therapeutics, i.e. compounds which are useful for treating and/or preventing the development of neoplastic conditions. In addition the assays are useful for further improving known

anti-proliferative compounds, e.g, by modifying their structure to increase their stability and/or activity and/or toxicity.

In an exemplary embodiment, the assay comprises administering a test compound to a transgenic animal of the invention, and comparing a phenotypic change, such as increased cellular proliferation in certain tissues, in the animal
5 relative to a transgenic animal which has not received the test compound.

Cells from the transgenic animals of the invention can be established in culture and immortalized to establish cell lines. For example, immortalized cell lines can be established from the livers of transgenic rats, as described in Bulera et al.
10 (1997) Hepatology 25: 1192. Cell lines from other types of cells can be established according to methods known in the art."

In one cell-based assay, cells expressing a POSH transgene can be incubated in the presence of a test compound or a control compound. The proliferation of cells is then compared. This assay system thus provides a means of identifying molecular
15 antagonists which, for example, function by interfering with cellular proliferation.

8. RNA Interference, Ribozymes, Antisense and DNA Enzyme

In certain aspects, the invention relates to RNAi, ribozyme, antisense and other nucleic acid-related methods and compositions for manipulating (typically
20 decreasing) a POSH activity. Exemplary RNAi and ribozyme molecules may comprise a sequence as shown in any of SEQ ID Nos: 15, 16, 18, 19, 21, 22, 24 and 25.

Certain embodiments of the invention make use of materials and methods for effecting knockdown of one or more POSH genes by means of RNA interference
25 (RNAi). RNAi is a process of sequence-specific post-transcriptional gene repression which can occur in eukaryotic cells. In general, this process involves degradation of an mRNA of a particular sequence induced by double-stranded RNA (dsRNA) that is homologous to that sequence. For example, the expression of a long dsRNA corresponding to the sequence of a particular single-stranded mRNA (ss mRNA)
30 will labilize that message, thereby "interfering" with expression of the corresponding gene. Accordingly, any selected gene may be repressed by

introducing a dsRNA which corresponds to all or a substantial part of the mRNA for that gene. It appears that when a long dsRNA is expressed, it is initially processed by a ribonuclease III into shorter dsRNA oligonucleotides of as few as 21 to 22 base pairs in length. Furthermore, Accordingly, RNAi may be effected by introduction or
5 expression of relatively short homologous dsRNAs. Indeed the use of relatively short homologous dsRNAs may have certain advantages as discussed below.

Mammalian cells have at least two pathways that are affected by double-stranded RNA (dsRNA). In the RNAi (sequence-specific) pathway, the initiating dsRNA is first broken into short interfering (si) RNAs, as described above. The
10 siRNAs have sense and antisense strands of about 21 nucleotides that form approximately 19 nucleotide si RNAs with overhangs of two nucleotides at each 3' end. Short interfering RNAs are thought to provide the sequence information that allows a specific messenger RNA to be targeted for degradation. In contrast, the nonspecific pathway is triggered by dsRNA of any sequence, as long as it is at least
15 about 30 base pairs in length. The nonspecific effects occur because dsRNA activates two enzymes: PKR, which in its active form phosphorylates the translation initiation factor eIF2 to shut down all protein synthesis, and 2', 5' oligoadenylate synthetase (2', 5'-AS), which synthesizes a molecule that activates Rnase L, a nonspecific enzyme that targets all mRNAs. The nonspecific pathway may
20 represents a host response to stress or viral infection, and, in general, the effects of the nonspecific pathway are preferably minimized under preferred methods of the present invention. Significantly, longer dsRNAs appear to be required to induce the nonspecific pathway and, accordingly, dsRNAs shorter than about 30 bases pairs are preferred to effect gene repression by RNAi (see Hunter et al. (1975) J Biol Chem
25 250: 409-17; Manche et al. (1992) Mol Cell Biol 12: 5239-48; Minks et al. (1979) J Biol Chem 254: 10180-3; and Elbashir et al. (2001) Nature 411: 494-8).

RNAi has been shown to be effective in reducing or eliminating the expression of a POSH gene in a number of different organisms including *Caenorhabditis elegans* (see e.g. Fire et al. (1998) Nature 391: 806-11), mouse eggs
30 and embryos (Wianny et al. (2000) Nature Cell Biol 2: 70-5; Svoboda et al. (2000) Development 127: 4147-56), and cultured RAT-1 fibroblasts (Bahramina et al. (1999) Mol Cell Biol 19: 274-83), and appears to be an anciently evolved pathway

available in eukaryotic plants and animals (Sharp (2001) *Genes Dev.* 15: 485-90). RNAi has proven to be an effective means of decreasing gene expression in a variety of cell types including HeLa cells, NIH/3T3 cells, COS cells, 293 cells and BHK-21 cells, and typically decreases expression of a gene to lower levels than that achieved
5 using antisense techniques and, indeed, frequently eliminates expression entirely (see Bass (2001) *Nature* 411: 428-9). In mammalian cells, siRNAs are effective at concentrations that are several orders of magnitude below the concentrations typically used in antisense experiments (Elbashir et al. (2001) *Nature* 411: 494-8).

The double stranded oligonucleotides used to effect RNAi are preferably less
10 than 30 base pairs in length and, more preferably, comprise about 25, 24, 23, 22, 21, 20, 19, 18 or 17 base pairs of ribonucleic acid. Optionally the dsRNA oligonucleotides of the invention may include 3' overhang ends. Exemplary 2-nucleotide 3' overhangs may be composed of ribonucleotide residues of any type and may even be composed of 2'-deoxythymidine residues, which lowers the cost of
15 RNA synthesis and may enhance nuclease resistance of siRNAs in the cell culture medium and within transfected cells (see Elbashi et al. (2001) *Nature* 411: 494-8). Longer dsRNAs of 50, 75, 100 or even 500 base pairs or more may also be utilized in certain embodiments of the invention. Exemplary concentrations of dsRNAs for effecting RNAi are about 0.05 nM, 0.1 nM, 0.5 nM, 1.0 nM, 1.5 nM, 25 nM or 100
20 nM, although other concentrations may be utilized depending upon the nature of the cells treated, the gene target and other factors readily discernable to the skilled artisan. Exemplary dsRNAs may be synthesized chemically or produced in vitro or in vivo using appropriate expression vectors. Exemplary synthetic RNAs include 21 nucleotide RNAs chemically synthesized using methods known in the art (e.g.
25 Expedite RNA phosphoramidites and thymidine phosphoramidite (Prologo, Germany). Synthetic oligonucleotides are preferably deprotected and gel-purified using methods known in the art (see e.g. Elbashir et al. (2001) *Genes Dev.* 15: 188-200). Longer RNAs may be transcribed from promoters, such as T7 RNA polymerase promoters, known in the art. A single RNA target, placed in both
30 possible orientations downstream of an in vitro promoter, will transcribe both strands of the target to create a dsRNA oligonucleotide of the desired target sequence. Any of the above RNA species will be designed to include a portion of

nucleic acid sequence represented in a POSH nucleic acid, such as, for example, a nucleic acid that hybridizes, under stringent and/or physiological conditions, to any of SEQ ID Nos: 1, 3, 4, 6, 8 and 10 and complements thereof.

The specific sequence utilized in design of the oligonucleotides may be any
5 contiguous sequence of nucleotides contained within the expressed gene message of the target. Programs and algorithms, known in the art, may be used to select appropriate target sequences. In addition, optimal sequences may be selected utilizing programs designed to predict the secondary structure of a specified single
10 stranded nucleic acid sequence and allowing selection of those sequences likely to occur in exposed single stranded regions of a folded mRNA. Methods and compositions for designing appropriate oligonucleotides may be found, for example, in U.S. Patent Nos. 6,251,588, the contents of which are incorporated herein by reference. Messenger RNA (mRNA) is generally thought of as a linear molecule
15 ribonucleotides, however studies have revealed a number of secondary and tertiary structures that exist in most mRNAs. Secondary structure elements in RNA are formed largely by Watson-Crick type interactions between different regions of the same RNA molecule. Important secondary structural elements include intramolecular double stranded regions, hairpin loops, bulges in duplex RNA and
20 internal loops. Tertiary structural elements are formed when secondary structural elements come in contact with each other or with single stranded regions to produce a more complex three dimensional structure. A number of researchers have measured the binding energies of a large number of RNA duplex structures and have derived a set of rules which can be used to predict the secondary structure of RNA
25 (see e.g. Jaeger et al. (1989) Proc. Natl. Acad. Sci. USA 86:7706 (1989); and Turner et al. (1988) Annu. Rev. Biophys. Biophys. Chem. 17:167) . The rules are useful in identification of RNA structural elements and, in particular, for identifying single stranded RNA regions which may represent preferred segments of the mRNA to target for silencing RNAi, ribozyme or antisense technologies. Accordingly,
30 preferred segments of the mRNA target can be identified for design of the RNAi mediating dsRNA oligonucleotides as well as for design of appropriate ribozyme and hammerheadribozyme compositions of the invention.

The dsRNA oligonucleotides may be introduced into the cell by transfection with an heterologous target gene using carrier compositions such as liposomes, which are known in the art- e.g. Lipofectamine 2000 (Life Technologies) as described by the manufacturer for adherent cell lines. Transfection of dsRNA oligonucleotides for targeting endogenous genes may be carried out using 5 Oligofectamine (Life Technologies). Transfection efficiency may be checked using fluorescence microscopy for mammalian cell lines after co-transfection of hGFP-encoding pAD3 (Kehlenback et al. (1998) J Cell Biol 141: 863-74). The effectiveness of the RNAi may be assessed by any of a number of assays following 10 introduction of the dsRNAs. These include Western blot analysis using antibodies which recognize the POSH gene product following sufficient time for turnover of the endogenous pool after new protein synthesis is repressed, reverse transcriptase polymerase chain reaction and Northern blot analysis to determine the level of existing POSH target mRNA.

15 Further compositions, methods and applications of RNAi technology are provided in U.S. Patent Application Nos. 6,278,039, 5,723,750 and 5,244,805, which are incorporated herein by reference.

Ribozyme molecules designed to catalytically cleave POSH mRNA transcripts can also be used to prevent translation of subject POSH mRNAs and/or 20 expression of POSH (see, e.g., PCT International Publication WO90/11364, published October 4, 1990; Sarver et al. (1990) Science 247:1222-1225 and U.S. Patent No. 5,093,246). Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. (For a review, see Rossi (1994) Current Biology 4: 469-471). The mechanism of ribozyme action involves sequence 25 specific hybridization of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage event. The composition of ribozyme molecules preferably includes one or more sequences complementary to a POSH mRNA, and the well known catalytic sequence responsible for mRNA cleavage or a functionally equivalent sequence (see, e.g., U.S. Pat. No. 5,093,246, which is 30 incorporated herein by reference in its entirety).

While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy target mRNAs, the use of hammerhead ribozymes is

preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. Preferably, the target mRNA has the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Haseloff and Gerlach ((1988) Nature 334:585-591; and see PCT Appln. No. WO89/05852, the contents of which are incorporated herein by reference). Hammerhead ribozyme sequences can be embedded in a stable RNA such as a transfer RNA (tRNA) to increase cleavage efficiency in vivo (Perriman et al. (1995) Proc. Natl. Acad. Sci. USA, 92: 6175-79; de Feyter, and Gaudron, Methods in Molecular Biology, Vol. 74, Chapter 43, "Expressing Ribozymes in Plants", Edited by Turner, P. C, Humana Press Inc., Totowa, N.J). In particular, RNA polymerase III-mediated expression of tRNA fusion ribozymes are well known in the art (see Kawasaki et al. (1998) Nature 393: 284-9; Kuwabara et al. (1998) Nature Biotechnol. 16: 961-5; and Kuwabara et al. (1998) Mol. Cell 2: 617-27; Koseki et al. (1999) J Virol 73: 1868-77; Kuwabara et al. (1999) Proc Natl Acad Sci USA 96: 1886-91; Tanabe et al. (2000) Nature 406: 473-4). There are typically a number of potential hammerhead ribozyme cleavage sites within a given target cDNA sequence. Preferably the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the target mRNA- to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts. Furthermore, the use of any cleavage recognition site located in the target sequence encoding different portions of the C-terminal amino acid domains of, for example, long and short forms of target would allow the selective targeting of one or the other form of the target, and thus, have a selective effect on one form of the target gene product.

Gene targeting ribozymes necessarily contain a hybridizing region complementary to two regions, each of at least 5 and preferably each 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 contiguous nucleotides in length of a POSH mRNA, such as an mRNA of a sequence represented in any of SEQ ID Nos: 1, 3, 4, 6, 8 or 10. In addition, ribozymes possess highly specific endoribonuclease activity, which autocatalytically cleaves the target sense mRNA. The present invention extends to ribozymes which hybridize to a sense mRNA encoding a POSH gene

such as a therapeutic drug target candidate gene, thereby hybridising to the sense mRNA and cleaving it, such that it is no longer capable of being translated to synthesize a functional polypeptide product.

The ribozymes of the present invention also include RNA endoribonucleases (hereinafter "Cech-type ribozymes") such as the one which occurs naturally in *Tetrahymena thermophila* (known as the IVS, or L-19 IVS RNA) and which has been extensively described by Thomas Cech and collaborators (Zaug, et al. (1984) *Science* 224:574-578; Zaug, et al. (1986) *Science* 231:470-475; Zaug, et al. (1986) *Nature* 324:429-433; published International patent application No. WO88/04300 by University Patents Inc.; Been, et al. (1986) *Cell* 47:207-216). The Cech-type ribozymes have an eight base pair active site which hybridizes to a target RNA sequence whereafter cleavage of the target RNA takes place. The invention encompasses those Cech-type ribozymes which target eight base-pair active site sequences that are present in a target gene or nucleic acid sequence.

Ribozymes can be composed of modified oligonucleotides (e.g., for improved stability, targeting, etc.) and should be delivered to cells which express the target gene in vivo. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous target messages and inhibit translation. Because ribozymes, unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

In certain embodiments, a ribozyme may be designed by first identifying a sequence portion sufficient to cause effective knockdown by RNAi. The same sequence portion may then be incorporated into a ribozyme. In this aspect of the invention, the gene-targeting portions of the ribozyme or RNAi are substantially the same sequence of at least 5 and preferably 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 or more contiguous nucleotides of a POSH nucleic acid, such as a nucleic acid of any of SEQ ID Nos: 1, 3, 4, 6, 8, or 10. In a long target RNA chain, significant numbers of target sites are not accessible to the ribozyme because they are hidden within secondary or tertiary structures (Birikh et al. (1997) *Eur J Biochem* 245: 1-16). To overcome the problem of target RNA accessibility,

computer generated predictions of secondary structure are typically used to identify targets that are most likely to be single-stranded or have an "open" configuration (see Jaeger et al. (1989) *Methods Enzymol* 183: 281-306). Other approaches utilize a systematic approach to predicting secondary structure which involves assessing a huge number of candidate hybridizing oligonucleotides molecules (see Milner et al. (1997) *Nat Biotechnol* 15: 537-41; and Patzel and Sczakiel (1998) *Nat Biotechnol* 16: 64-8). Additionally, U.S. Patent No. 6,251,588, the contents of which are hereby incorporated herein, describes methods for evaluating oligonucleotide probe sequences so as to predict the potential for hybridization to a target nucleic acid sequence. The method of the invention provides for the use of such methods to select preferred segments of a target mRNA sequence that are predicted to be single-stranded and, further, for the opportunistic utilization of the same or substantially identical target mRNA sequence, preferably comprising about 10-20 consecutive nucleotides of the target mRNA, in the design of both the RNAi oligonucleotides and ribozymes of the invention.

A further aspect of the invention relates to the use of the isolated "antisense" nucleic acids to inhibit expression, e.g., by inhibiting transcription and/or translation of a subject POSH nucleic acid. The antisense nucleic acids may bind to the potential drug target by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, these methods refer to the range of techniques generally employed in the art, and include any methods that rely on specific binding to oligonucleotide sequences.

An antisense construct of the present invention can be delivered, for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the cellular mRNA which encodes a POSH polypeptide. Alternatively, the antisense construct is an oligonucleotide probe, which is generated ex vivo and which, when introduced into the cell causes inhibition of expression by hybridizing with the mRNA and/or genomic sequences of a POSH nucleic acid. Such oligonucleotide probes are preferably modified oligonucleotides, which are resistant to endogenous nucleases, e.g., exonucleases and/or endonucleases, and are therefore stable in vivo.

Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see also U.S. Patents 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by Van der Krol et al. (1988) *BioTechniques* 6:958-976; and Stein et al. (1988) *Cancer Res* 48:2659-2668.

With respect to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, e.g., between the -10 and +10 regions of the POSH gene, are preferred. Antisense approaches involve the design of oligonucleotides (either DNA or RNA) that are complementary to mRNA encoding the POSH polypeptide. The antisense oligonucleotides will bind to the mRNA transcripts and prevent translation. Absolute complementarity, although preferred, is not required. In the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

Oligonucleotides that are complementary to the 5' end of the mRNA, e.g., the 5' untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have recently been shown to be effective at inhibiting translation of mRNAs as well. (Wagner, R. 1994. *Nature* 372:333). Therefore, oligonucleotides complementary to either the 5' or 3' untranslated, non-coding regions of a gene could be used in an antisense approach to inhibit translation of that mRNA. Oligonucleotides complementary to the 5' untranslated region of the mRNA should include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could also be used in accordance with the invention. Whether designed to hybridize to the 5', 3' or coding region of mRNA, antisense

nucleic acids should be at least six nucleotides in length, and are preferably less than about 100 and more preferably less than about 50, 25, 17 or 10 nucleotides in length.

It is preferred that in vitro studies are first performed to quantitate the ability of the antisense oligonucleotide to inhibit gene expression. It is preferred that these studies utilize controls that distinguish between antisense gene inhibition and nonspecific biological effects of oligonucleotides. It is also preferred that these studies compare levels of the target RNA or protein with that of an internal control RNA or protein. Results obtained using the antisense oligonucleotide may be compared with those obtained using a control oligonucleotide. It is preferred that the control oligonucleotide is of approximately the same length as the test oligonucleotide and that the nucleotide sequence of the oligonucleotide differs from the antisense sequence no more than is necessary to prevent specific hybridization to the target sequence.

The antisense oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, etc. The oligonucleotide may include other appended groups such as peptides (e.g., for targeting host cell receptors), or agents facilitating transport across the cell membrane (see, e.g., Letsinger et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:6553-6556; Lemaitre et al., 1987, Proc. Natl. Acad. Sci. 84:648-652; PCT Publication No. W088/09810, published December 15, 1988) or the blood-brain barrier (see, e.g., PCT Publication No. W089/10134, published April 25, 1988), hybridization-triggered cleavage agents. (See, e.g., Krol et al., 1988, BioTechniques 6:958-976) or intercalating agents. (See, e.g., Zon, 1988, Pharm. Res. 5:539-549). To this end, the oligonucleotide may be conjugated to another molecule, e.g., a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxyethyl) uracil, 5-carboxymethylaminomethyl-2-

thiouridine, 5- carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6- isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-
 5 methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6- isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5- oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-
 10 methyl-2-thiouracil, 3-(3-amino-3- N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

15 The antisense oligonucleotide can also contain a neutral peptide-like backbone. Such molecules are termed peptide nucleic acid (PNA)-oligomers and are described, e.g., in Perry-O'Keefe et al. (1996) Proc. Natl. Acad. Sci. U.S.A. 93:14670 and in Eglom et al. (1993) Nature 365:566. One advantage of PNA oligomers is their capability to bind to complementary DNA essentially
 20 independently from the ionic strength of the medium due to the neutral backbone of the DNA. In yet another embodiment, the antisense oligonucleotide comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl
 25 phosphotriester, and a formacetal or analog thereof.

In yet a further embodiment, the antisense oligonucleotide is an alpha-anomeric oligonucleotide. An alpha-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual antiparallel orientation, the strands run parallel to each other (Gautier et al., 1987, Nucl. Acids Res. 15:6625-6641). The oligonucleotide is a 2'-O-methylribonucleotide
 30 (Inoue et al., 1987, Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al., 1987, FEBS Lett. 215:327-330).

While antisense nucleotides complementary to the coding region of a POSH mRNA sequence can be used, those complementary to the transcribed untranslated region may also be used.

In certain instances, it may be difficult to achieve intracellular concentrations of the antisense sufficient to suppress translation on endogenous mRNAs. Therefore a preferred approach utilizes a recombinant DNA construct in which the antisense oligonucleotide is placed under the control of a strong pol III or pol II promoter. The use of such a construct to transfect target cells will result in the transcription of sufficient amounts of single stranded RNAs that will form complementary base pairs with the endogenous potential drug target transcripts and thereby prevent translation. For example, a vector can be introduced such that it is taken up by a cell and directs the transcription of an antisense RNA. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the antisense RNA can be by any promoter known in the art to act in mammalian, preferably human cells. Such promoters can be inducible or constitutive. Such promoters include but are not limited to: the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al, 1982, Nature 296:39-42), etc. Any type of plasmid, cosmid, YAC or viral vector can be used to prepare the recombinant DNA construct, which can be introduced directly into the tissue site.

Alternatively, POSH gene expression can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the gene (i.e., the promoter and/or enhancers) to form triple helical structures that prevent transcription of the gene in target cells in the body. (See generally, Helene, C. 1991, Anticancer Drug Des., 6(6):569-84; Helene, C., et al., 1992, Ann. N.Y. Acad. Sci., 660:27-36; and Maher, L.J., 1992, Bioassays 14(12):807-15).

Nucleic acid molecules to be used in triple helix formation for the inhibition of transcription are preferably single stranded and composed of deoxyribonucleotides. The base composition of these oligonucleotides should promote triple helix formation via Hoogsteen base pairing rules, which generally require sizable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine- rich, for example, containing a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in CGC triplets across the three strands in the triplex.

Alternatively, the potential POSH sequences that can be targeted for triple helix formation may be increased by creating a so called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizable stretch of either purines or pyrimidines to be present on one strand of a duplex.

A further aspect of the invention relates to the use of DNA enzymes to inhibit expression of POSH gene. DNA enzymes incorporate some of the mechanistic features of both antisense and ribozyme technologies. DNA enzymes are designed so that they recognize a particular target nucleic acid sequence, much like an antisense oligonucleotide, however much like a ribozyme they are catalytic and specifically cleave the target nucleic acid.

There are currently two basic types of DNA enzymes, and both of these were identified by Santoro and Joyce (see, for example, US Patent No. 6110462). The 10-23 DNA enzyme comprises a loop structure which connect two arms. The two arms provide specificity by recognizing the particular target nucleic acid sequence while the loop structure provides catalytic function under physiological conditions.

Briefly, to design an ideal DNA enzyme that specifically recognizes and cleaves a target nucleic acid, one of skill in the art must first identify the unique target sequence. This can be done using the same approach as outlined for antisense oligonucleotides. Preferably, the unique or substantially sequence is a G/C rich of
5 approximately 18 to 22 nucleotides. High G/C content helps insure a stronger interaction between the DNA enzyme and the target sequence.

When synthesizing the DNA enzyme, the specific antisense recognition sequence that will target the enzyme to the message is divided so that it comprises the two arms of the DNA enzyme, and the DNA enzyme loop is placed between the
10 two specific arms.

Methods of making and administering DNA enzymes can be found, for example, in US 6110462. Similarly, methods of delivery DNA ribozymes in vitro or in vivo include methods of delivery RNA ribozyme, as outlined in detail above. Additionally, one of skill in the art will recognize that, like antisense
15 oligonucleotide, DNA enzymes can be optionally modified to improve stability and improve resistance to degradation.

Antisense RNA and DNA, ribozyme, RNAi and triple helix molecules of the invention may be prepared by any method known in the art for the synthesis of DNA and RNA molecules. These include techniques for chemically synthesizing
20 oligodeoxyribonucleotides and oligoribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by in vitro and in vivo transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase
25 promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines. Moreover, various well-known modifications to nucleic acid molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications
30 include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3' ends of the molecule or the use of

phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

9. Drug Screening Assays

5 In certain aspects, the present invention also provides assays for identifying therapeutic agents which either interfere with or promote POSH function. In certain embodiments, agents of the invention are antiproliferative agents, and optionally such agents are useful, alone or in combination with other therapeutics, for treatment of neoplastic conditions, including benign tumors and malignant tumors, i.e.,
10 cancers. In a preferred embodiment, agents of the invention are useful for treating cancers including thyroid carcinoma, liver cancer (hepatocellular cancer), lung cancer, cervical cancer and ovarian cancer. In another preferred embodiment, agents of the invention are useful for treating cancers including renal cell carcinoma, lymphoma, osteosarcoma, liposarcoma, leukemia, breast carcinoma, breast adeno-
15 carcinoma. In an additional embodiment, agents of the invention are useful for treating cancers including papillomas (e.g. HPV-related papillomas, condylomata acuminatum), cervical squamous intraepithelial lesions, oral cancer, penile cancer, clear cell sarcoma, etc. (Jishage, M et al (2003) Oncogene 22:41-49). In yet other embodiment, agents of the invention are pro-proliferative. Pro-proliferative agents
20 may be useful in a variety of situations, such as wound healing and angiogenesis.

 In certain preferred embodiments, an antiproliferative agent interferes with the ubiquitin ligase catalytic activity of POSH (e.g. POSH auto-ubiquitination or transfer to a target protein). In certain preferred embodiments, an antiproliferative agent interferes with the interaction between POSH and a POSH-AP polypeptide,
25 for example an antiproliferative agent may disrupt or render irreversible the

interaction between a POSH polypeptide and POSH-AP polypeptide such as another POSH polypeptide (as in the case of a POSH dimer, a heterodimer of two different POSH polypeptides, homomultimers and heteromultimers); a GTPase (eg. Rac, Rac1, Rho, Ras); an E2 enzyme and ubiquitin, or optionally, a cullin; a clathrin; AP-1; AP-2; an HSP70; an HSP90, Bcr1, Bard1, Nef, PAK1, PAK2, PAK family, Vav, Cdc42, PI3K (e.g. p85 or p110), Nedd4, src (src family), a Gag, Tsg101, VASP, RNB6, WASP, N-WASP and KIAA0674, Similar to Spred-2, as well as, in certain embodiments, proteins known to be associated with clathrin-coated vesicles and or proteins involved in the protein sorting pathway. In further embodiments, agents of the invention are anti-apoptotic agents, optionally interfering with JNK and/or NF- κ B signaling. In yet additional embodiments, agents of the invention interfere with the signaling of a GTPase, such as Rac or Ras, optionally disrupting the interaction between a POSH polypeptide and a Rac protein. In certain embodiments, agents of the invention modulate the ubiquitin ligase activity of POSH and may be used to treat certain diseases related to ubiquitin ligase activity.

In certain embodiments, the invention provides assays to identify, optimize or otherwise assess agents that increase or decrease a ubiquitin-related activity of a POSH polypeptide. Ubiquitin-related activities of POSH polypeptides may include the self-ubiquitination activity of a POSH polypeptide, generally involving the transfer of ubiquitin from an E2 enzyme to the POSH polypeptide, and the ubiquitination of a target protein, generally involving the transfer of a ubiquitin from a POSH polypeptide to the target protein. In certain embodiments, a POSH activity is mediated, at least in part, by a POSH RING domain.

In certain embodiments, an assay comprises forming a mixture comprising a POSH polypeptide, an E2 polypeptide and a source of ubiquitin (which may be the E2 polypeptide pre-complexed with ubiquitin). Optionally the mixture comprises an

E1 polypeptide and optionally the mixture comprises a target polypeptide. Additional components of the mixture may be selected to provide conditions consistent with the ubiquitination of the POSH polypeptide. One or more of a variety of parameters may be detected, such as POSH-ubiquitin conjugates, E2-ubiquitin thioesters, free ubiquitin and target polypeptide-ubiquitin complexes. The term "detect" is used herein to include a determination of the presence or absence of the subject of detection (e.g. POSH-ubiquitin, E2-ubiquitin, etc.), a quantitative measure of the amount of the subject of detection, or a mathematical calculation of the presence, absence or amount of the subject of detection, based on the detection of other parameters. The term "detect" includes the situation wherein the subject of detection is determined to be absent or below the level of sensitivity. Detection may comprise detection of a label (e.g. fluorescent label, radioisotope label, and other described below), resolution and identification by size (e.g. SDS-PAGE, mass spectroscopy), purification and detection, and other methods that, in view of this specification, will be available to one of skill in the art. For instance, radioisotope labeling may be measured by scintillation counting, or by densitometry after exposure to a photographic emulsion, or by using a device such as a Phosphorimager. Likewise, densitometry may be used to measure bound ubiquitin following a reaction with an enzyme label substrate that produces an opaque product when an enzyme label is used. In a preferred embodiment, an assay comprises detecting the POSH-ubiquitin conjugate.

In certain embodiments, an assay comprises forming a mixture comprising a POSH polypeptide, a target polypeptide and a source of ubiquitin (which may be the POSH polypeptide pre-complexed with ubiquitin). Optionally the mixture comprises an E1 and/or E2 polypeptide and optionally the mixture comprises an E2-ubiquitin thioester. Additional components of the mixture may be selected to provide conditions consistent with the ubiquitination of the target polypeptide. One or more of a variety of parameters may be detected, such as POSH-ubiquitin conjugates and target polypeptide-ubiquitin conjugates. In a preferred embodiment, an assay comprises detecting the target polypeptide-ubiquitin conjugate. In another preferred embodiment, an assay comprises detecting the POSH-ubiquitin conjugate.

An assay described above may be used in a screening assay to identify agents that modulate a ubiquitin-related activity of a POSH polypeptide. A screening assay will generally involve adding a test agent to one of the above assays, or any other assay designed to assess a ubiquitin-related activity of a POSH polypeptide. The
5 parameter(s) detected in a screening assay may be compared to a suitable reference. A suitable reference may be an assay run previously, in parallel or later that omits the test agent. A suitable reference may also be an average of previous measurements in the absence of the test agent. In general the components of a screening assay mixture may be added in any order consistent with the overall
10 activity to be assessed, but certain variations may be preferred. For example, in certain embodiments, it may be desirable to pre-incubate the test agent and the E3 (e.g. the POSH polypeptide), followed by removing the test agent and addition of other components to complete the assay. In this manner, the effects of the agent solely on the POSH polypeptide may be assessed..

15 In certain embodiments, an assay is performed in a high-throughput format. For example, one of the components of a mixture may be affixed to a solid substrate and one or more of the other components is labeled. For example, the POSH polypeptide may be affixed to a surface, such as a 96-well plate, and the ubiquitin is in solution and labeled. An E2 and E1 are also in solution, and the POSH-ubiquitin
20 conjugate formation may be measured by washing the solid surface to remove uncomplexed labeled ubiquitin and detecting the ubiquitin that remains bound. Other variations may be used. For example, the amount of ubiquitin in solution may be detected. In certain embodiments, the formation of ubiquitin complexes may be measured by an interactive technique, such as FRET, wherein a ubiquitin is labeled
25 with a first label and the desired complex partner (e.g. POSH polypeptide or target polypeptide) is labeled with a second label, wherein the first and second label interact when they come into close proximity to produce an altered signal. In FRET, the first and second labels are fluorophores. FRET is described in greater detail below. The formation of polyubiquitin complexes may be performed by
30 mixing two or more pools of differentially labeled ubiquitin that interact upon formation of a polyubiquitin (see, e.g. US Patent Publication 20020042083). High-throughput may be achieved by performing an interactive assay, such as FRET, in

solution as well. In addition, if a polypeptide in the mixture, such as the POSH polypeptide or target polypeptide, is readily purifiable (e.g. with a specific antibody or via a tag such as biotin, FLAG, polyhistidine, etc.), the reaction may be performed in solution and the tagged polypeptide rapidly isolated, along with any
5 polypeptides, such as ubiquitin, that are associated with the tagged polypeptide. Proteins may also be resolved by SDS-PAGE for detection.

In certain embodiments, the ubiquitin is labeled, either directly or indirectly. This typically allows for easy and rapid detection and measurement of ligated ubiquitin, making the assay useful for high-throughput screening applications. As
10 described above, certain embodiments may employ one or more tagged or labeled proteins. A "tag" is meant to include moieties that facilitate rapid isolation of the tagged polypeptide. A tag may be used to facilitate attachment of a polypeptide to a surface. A "label" is meant to include moieties that facilitate rapid detection of the labeled polypeptide. Certain moieties may be used both as a label and a tag (e.g.
15 epitope tags that are readily purified and detected with a well-characterized antibody). Biotinylation of polypeptides is well known, for example, a large number of biotinylation agents are known, including amine-reactive and thiol-reactive agents, for the biotinylation of proteins, nucleic acids, carbohydrates, carboxylic acids; see chapter 4, Molecular Probes Catalog, Haugland, 6th Ed. 1996, hereby
20 incorporated by reference. A biotinylated substrate can be attached to a biotinylated component via avidin or streptavidin. Similarly, a large number of haptenylation reagents are also known.

An "E1" is a ubiquitin activating enzyme. In a preferred embodiment, E1 is capable of transferring ubiquitin to an E2. In a preferred embodiment, E1 forms a
25 high energy thiolester bond with ubiquitin, thereby "activating" the ubiquitin. An "E2" is a ubiquitin carrier enzyme (also known as a ubiquitin conjugating enzyme). In a preferred embodiment, ubiquitin is transferred from E1 to E2. In a preferred embodiment, the transfer results in a thiolester bond formed between E2 and ubiquitin. In a preferred embodiment, E2 is capable of transferring ubiquitin to a
30 POSH polypeptide.

In an alternative embodiment, a POSH polypeptide, E2 or target polypeptide is bound to a bead, optionally with the assistance of a tag. Following ligation, the beads may be separated from the unbound ubiquitin and the bound ubiquitin measured. In a preferred embodiment, POSH polypeptide is bound to beads and the composition used includes labeled ubiquitin. In this embodiment, the beads with bound ubiquitin may be separated using a fluorescence-activated cell sorting (FACS) machine. Methods for such use are described in U.S. patent application Ser. No. 09/047,119, which is hereby incorporated in its entirety. The amount of bound ubiquitin can then be measured.

In a screening assay, the effect of a test agent may be assessed by, for example, assessing the effect of the test agent on kinetics, steady-state and/or endpoint of the reaction.

The components of the various assay mixtures provided herein may be combined in varying amounts. In a preferred embodiment, ubiquitin (or E2 complexed ubiquitin) is combined at a final concentration of from 5 to 200 ng per 100 microliter reaction solution. Optionally E1 is used at a final concentration of from 1 to 50 ng per 100 microliter reaction solution. Optionally E2 is combined at a final concentration of 10 to 100 ng per 100 microliter reaction solution, more preferably 10-50 ng per 100 microliter reaction solution. In a preferred embodiment, POSH polypeptide is combined at a final concentration of from 1 ng to 500 ng per 100 microliter reaction solution.

Generally, an assay mixture is prepared so as to favor ubiquitin ligase activity and/or ubiquitination activity. Generally, this will be physiological conditions, such as 50 – 200 mM salt (e.g. NaCl, KCl), pH of between 5 and 9, and preferably between 6 and 8. Such conditions may be optimized through trial and error. Incubations may be performed at any temperature which facilitates optimal activity, typically between 4 and 40 degrees C. Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high throughput screening. Typically between 0.5 and 1.5 hours will be sufficient. A variety of other reagents may be included in the compositions. These include reagents like salts,

solvents, buffers, neutral proteins, e.g. albumin, detergents, etc. which may be used to facilitate optimal ubiquitination enzyme activity and/or reduce non-specific or background interactions. Also reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc.,
5 may be used. The compositions will also preferably include adenosine tri-phosphate (ATP). The mixture of components may be added in any order that promotes ubiquitin ligase activity or optimizes identification of candidate modulator effects. In a preferred embodiment, ubiquitin is provided in a reaction buffer solution, followed by addition of the ubiquitination enzymes. In an alternate preferred embodiment,
10 ubiquitin is provided in a reaction buffer solution, a candidate modulator is then added, followed by addition of the ubiquitination enzymes.

In general, a test agent that decreases a POSH ubiquitin-related activity may be used to inhibit POSH function in vivo, while a test agent that increases a POSH ubiquitin-related activity may be used to stimulate POSH function in vivo. Test
15 agent may be modified for use in vivo, e.g. by addition of a hydrophobic moiety, such as an ester.

Certain embodiments of the invention relate to assays for identifying agents that bind to a POSH polypeptide, optionally a particular domain of POSH such as an SH3 or RING domain. In preferred embodiments, a POSH polypeptide is a
20 polypeptide comprising the fourth SH3 domain of hPOSH (SEQ ID NO: 30). A wide variety of assays may be used for this purpose, including labeled in vitro protein-protein binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, and the like. The purified protein may also be used for determination of three-dimensional crystal structure, which can be used for
25 modeling intermolecular interactions and design of test agents. In one embodiment, an assay detects agents which inhibit interaction of one or more subject POSH polypeptides with a POSH-AP. In another embodiment, the assay detects agents which modulate the intrinsic biological activity of a POSH polypeptide or POSH complex, such as an enzymatic activity, binding to other cellular components,
30 cellular compartmentalization, and the like.

In one aspect, the invention provides methods and compositions for the identification of compositions that interfere with the function of POSH polypeptides. Given the role of POSH polypeptides in cellular proliferation, compositions that perturb the formation or stability of the protein-protein interactions between POSH polypeptides and the proteins that they interact with, such as POSH-APs, are candidate pharmaceuticals for the treatment of neoplastic conditions, including benign and malignant tumors.

While not wishing to be bound to mechanism, it is postulated that POSH polypeptides promote the assembly of protein complexes that are important in cellular proliferation and other biological processes. Complexes of the invention may include a combination of a POSH polypeptide and one or more of the following POSH-APs: a POSH-AP; a POSH polypeptide (as in the case of a POSH dimer, a heterodimer of two different POSH, homomultimers and heteromultimers); a GTPase (eg. Rac, Rac1, Rho, Ras); an E2 enzyme; ubiquitin, or optionally, a cullin; a clathrin; AP-1; AP-2; an HSP70; an HSP90, Brca1, Bard1, Nef, PAK1, PAK2, PAK family, Vav, Cdc42, PI3K (e.g. p85 or p110), Nedd4, src (src family), Tsg101, VASP, RNB6, WASP, N-WASP, a Gag, and KIAA0674, Similar to Spred-2, as well as, in certain embodiments, proteins known to be associated with clathrin-coated vesicles and or proteins involved in the protein sorting pathway.

The type of complex formed by a POSH polypeptide will depend upon the domains present in the protein. While not intended to be limiting, exemplary domains of potential interacting proteins are provided below. A RING domain is expected to interact with cullins, E2 enzymes, AP-1, AP-2, and/or a substrate for ubiquitylation (e.g. in some instances, a protein comprising a Gag L domain). An SH3 domain may interact with Gag L domains and other proteins having the sequence motif P(T/S)AP, RXXP(T/S)AP, PXXDY, PXXP, PPXY or RXXPXXP, such as, for example, an HIV Gag sequence such as RQGPKEPFR, PFRDY, PTAP and RPEPTAP.

In a preferred assay for an antiproliferative or antiapoptotic agent, the test agent is assessed for its ability to disrupt or inhibit the formation of a complex of a POSH polypeptide and a Rac polypeptide, particularly a human Rac polypeptide, such as Rac1.

A variety of assay formats will suffice and, in light of the present disclosure, those not expressly described herein will nevertheless be comprehended by one of ordinary skill in the art. Assay formats which approximate such conditions as formation of protein complexes, enzymatic activity, and even a POSH polypeptide-mediated membrane reorganization or vesicle formation activity, may be generated
5 in many different forms, and include assays based on cell-free systems, e.g. purified proteins or cell lysates, as well as cell-based assays which utilize intact cells. Simple binding assays can also be used to detect agents which bind to POSH. Such binding assays may also identify agents that act by disrupting the interaction
10 between a POSH polypeptide and a POSH interacting protein, or the binding of a POSH polypeptide or complex to a substrate. Agents to be tested can be produced, for example, by bacteria, yeast or other organisms (e.g. natural products), produced chemically (e.g. small molecules, including peptidomimetics), or produced recombinantly. In a preferred embodiment, the test agent is a small organic
15 molecule, e.g., other than a peptide or oligonucleotide, having a molecular weight of less than about 2,000 daltons.

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays of the present
20 invention which are performed in cell-free systems, such as may be developed with purified or semi-purified proteins or with lysates, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the
25 test compound can be generally ignored in the in vitro system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an alteration of binding affinity with other proteins or changes in enzymatic properties of the molecular target.

In preferred in vitro embodiments of the present assay, a reconstituted POSH
30 complex comprises a reconstituted mixture of at least semi-purified proteins. By semi-purified, it is meant that the proteins utilized in the reconstituted mixture have been previously separated from other cellular or viral proteins. For instance, in

contrast to cell lysates, the proteins involved in POSH complex formation are present in the mixture to at least 50% purity relative to all other proteins in the mixture, and more preferably are present at 90-95% purity. In certain embodiments of the subject method, the reconstituted protein mixture is derived by mixing highly purified proteins such that the reconstituted mixture substantially lacks other proteins (such as of cellular or viral origin) which might interfere with or otherwise alter the ability to measure POSH complex assembly and/or disassembly.

Assaying POSH complexes, in the presence and absence of a candidate inhibitor, can be accomplished in any vessel suitable for containing the reactants. Examples include microtitre plates, test tubes, and micro-centrifuge tubes.

In one embodiment of the present invention, drug screening assays can be generated which detect inhibitory agents on the basis of their ability to interfere with assembly or stability of the POSH complex. In an exemplary binding assay, the compound of interest is contacted with a mixture comprising a POSH polypeptide and at least one interacting polypeptide. Detection and quantification of POSH complexes provides a means for determining the compound's efficacy at inhibiting (or potentiating) interaction between the two polypeptides. The efficacy of the compound can be assessed by generating dose response curves from data obtained using various concentrations of the test compound. Moreover, a control assay can also be performed to provide a baseline for comparison. In the control assay, the formation of complexes is quantitated in the absence of the test compound.

Complex formation between the POSH polypeptides and a substrate polypeptide may be detected by a variety of techniques, many of which are effectively described above. For instance, modulation in the formation of complexes can be quantitated using, for example, detectably labeled proteins (e.g. radiolabeled, fluorescently labeled, or enzymatically labeled), by immunoassay, or by chromatographic detection. Surface plasmon resonance systems, such as those available from Biacore International AB (Uppsala, Sweden), may also be used to detect protein-protein interaction

Often, it will be desirable to immobilize one of the polypeptides to facilitate separation of complexes from uncomplexed forms of one of the proteins, as well as to accommodate automation of the assay. In an illustrative embodiment, a fusion

protein can be provided which adds a domain that permits the protein to be bound to an insoluble matrix. For example, GST-POSH fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with a potential interacting
5 protein, e.g. an ³⁵S-labeled polypeptide, and the test compound and incubated under conditions conducive to complex formation. Following incubation, the beads are washed to remove any unbound interacting protein, and the matrix bead-bound radiolabel determined directly (e.g. beads placed in scintillant), or in the supernatant after the complexes are dissociated, e.g. when microtitre plate is used. Alternatively,
10 after washing away unbound protein, the complexes can be dissociated from the matrix, separated by SDS-PAGE gel, and the level of interacting polypeptide found in the matrix-bound fraction quantitated from the gel using standard electrophoretic techniques.

In a further embodiment, agents that bind to a POSH may be identified by
15 using an immobilized POSH. In an illustrative embodiment, a fusion protein can be provided which adds a domain that permits the protein to be bound to an insoluble matrix. For example, GST-POSH fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with a potential labeled binding agent
20 and incubated under conditions conducive to binding. Following incubation, the beads are washed to remove any unbound agent, and the matrix bead-bound label determined directly, or in the supernatant after the bound agent is dissociated.

In yet another embodiment, the POSH polypeptide and potential interacting polypeptide can be used to generate an interaction trap assay (see also, U.S. Patent
25 NO: 5,283,317; Zervos et al. (1993) Cell 72:223-232; Madura et al. (1993) J Biol Chem 268:12046-12054; Bartel et al. (1993) Biotechniques 14:920-924; and Iwabuchi et al. (1993) Oncogene 8:1693-1696), for subsequently detecting agents which disrupt binding of the proteins to one and other.

In particular, the method makes use of chimeric genes which express hybrid
30 proteins. To illustrate, a first hybrid gene comprises the coding sequence for a DNA-binding domain of a transcriptional activator can be fused in frame to the coding sequence for a "bait" protein, e.g., a POSH polypeptide of sufficient length to

bind to a potential interacting protein. The second hybrid protein encodes a transcriptional activation domain fused in frame to a gene encoding a "fish" protein, e.g., a potential interacting protein of sufficient length to interact with the POSH polypeptide portion of the bait fusion protein. If the bait and fish proteins are able to interact, e.g., form a POSH complex, they bring into close proximity the two domains of the transcriptional activator. This proximity causes transcription of a reporter gene which is operably linked to a transcriptional regulatory site responsive to the transcriptional activator, and expression of the reporter gene can be detected and used to score for the interaction of the bait and fish proteins.

10 In accordance with the present invention, the method includes providing a host cell, preferably a yeast cell, e.g., *Kluyveri lactis*, *Schizosaccharomyces pombe*, *Ustilago maydis*, *Saccharomyces cerevisiae*, *Neurospora crassa*, *Aspergillus niger*, *Aspergillus nidulans*, *Pichia pastoris*, *Candida tropicalis*, and *Hansenula polymorpha*, though most preferably *S. cerevisiae* or *S. pombe*. The host cell contains a reporter gene having a binding site for the DNA-binding domain of a transcriptional activator used in the bait protein, such that the reporter gene expresses a detectable gene product when the gene is transcriptionally activated. The first chimeric gene may be present in a chromosome of the host cell, or as part of an expression vector. Interaction trap assays may also be performed in mammalian and bacterial cell types.

The host cell also contains a first chimeric gene which is capable of being expressed in the host cell. The gene encodes a chimeric protein, which comprises (i) a DNA-binding domain that recognizes the responsive element on the reporter gene in the host cell, and (ii) a bait protein, such as a POSH polypeptide sequence.

25 A second chimeric gene is also provided which is capable of being expressed in the host cell, and encodes the "fish" fusion protein. In one embodiment, both the first and the second chimeric genes are introduced into the host cell in the form of plasmids. Preferably, however, the first chimeric gene is present in a chromosome of the host cell and the second chimeric gene is introduced into the host cell as part of a plasmid.

30 Preferably, the DNA-binding domain of the first hybrid protein and the transcriptional activation domain of the second hybrid protein are derived from

transcriptional activators having separable DNA-binding and transcriptional activation domains. For instance, these separate DNA-binding and transcriptional activation domains are known to be found in the yeast GAL4 protein, and are known to be found in the yeast GCN4 and ADR1 proteins. Many other proteins involved in transcription also have separable binding and transcriptional activation domains which make them useful for the present invention, and include, for example, the LexA and VP16 proteins. It will be understood that other (substantially) transcriptionally-inert DNA-binding domains may be used in the subject constructs; such as domains of ACE1, Icl, lac repressor, jun or fos. In another embodiment, the DNA-binding domain and the transcriptional activation domain may be from different proteins. The use of a LexA DNA binding domain provides certain advantages. For example, in yeast, the LexA moiety contains no activation function and has no known effect on transcription of yeast genes. In addition, use of LexA allows control over the sensitivity of the assay to the level of interaction (see, for example, the Brent et al. PCT publication WO94/10300).

In preferred embodiments, any enzymatic activity associated with the bait or fish proteins is inactivated, e.g., dominant negative or other mutants of a POSH polypeptide can be used.

Continuing with the illustrated example, the POSH polypeptide-mediated interaction, if any, between the bait and fish fusion proteins in the host cell, therefore, causes the activation domain to activate transcription of the reporter gene. The method is carried out by introducing the first chimeric gene and the second chimeric gene into the host cell, and subjecting that cell to conditions under which the bait and fish fusion proteins are expressed in sufficient quantity for the reporter gene to be activated. The formation of a POSH - POSH-AP complex results in a detectable signal produced by the expression of the reporter gene. Accordingly, the level of formation of a complex in the presence of a test compound and in the absence of the test compound can be evaluated by detecting the level of expression of the reporter gene in each case. Various reporter constructs may be used in accord with the methods of the invention and include, for example, reporter genes which produce such detectable signals as selected from the group consisting of an enzymatic signal, a fluorescent signal, a phosphorescent signal and drug resistance.

One aspect of the present invention provides reconstituted protein preparations including a POSH polypeptide and one or more interacting polypeptides.

In still further embodiments of the present assay, the POSH complex is generated in whole cells, taking advantage of cell culture techniques to support the subject assay. For example, as described below, the POSH complex can be constituted in a eukaryotic cell culture system, including mammalian and yeast cells. Advantages to generating the subject assay in an intact cell include the ability to detect inhibitors which are functional in an environment more closely approximating that which therapeutic use of the inhibitor would require, including the ability of the agent to gain entry into the cell. Furthermore, certain of the in vivo embodiments of the assay, such as examples given below, are amenable to high through-put analysis of candidate agents.

The components of the POSH complex can be endogenous to the cell selected to support the assay. Alternatively, some or all of the components can be derived from exogenous sources. For instance, fusion proteins can be introduced into the cell by recombinant techniques (such as through the use of an expression vector), as well as by microinjecting the fusion protein itself or mRNA encoding the fusion protein.

In many embodiments, a cell is manipulated after incubation with a candidate agent and assayed for a POSH activity. In certain embodiments a POSH activity is represented by cellular proliferation. As demonstrated herein, an agent that reduces POSH activity can cause a decrease in cellular proliferation in cancer cells (for example, in Hela cells). Other bioassays for POSH activities may include apoptosis assays (e.g. cell survival assays, apoptosis reporter gene assays, etc.) and NF-kB nuclear localization assays (see e.g. Tapon et al. (1998) EMBO J. 17: 1395-1404). In certain embodiments, POSH activities may include, without limitation, complex formation, ubiquitination and membrane fusion events. POSH complex formation may be assessed by immunoprecipitation and analysis of co-immunoprecipitated proteins or affinity purification and analysis of co-purified proteins. Fluorescence Resonance Energy Transfer (FRET)-based assays may also be used to determine complex formation. Fluorescent molecules having the proper

emission and excitation spectra that are brought into close proximity with one another can exhibit FRET. The fluorescent molecules are chosen such that the emission spectrum of one of the molecules (the donor molecule) overlaps with the excitation spectrum of the other molecule (the acceptor molecule). The donor molecule is excited by light of appropriate intensity within the donor's excitation spectrum. The donor then emits the absorbed energy as fluorescent light. The fluorescent energy it produces is quenched by the acceptor molecule. FRET can be manifested as a reduction in the intensity of the fluorescent signal from the donor, reduction in the lifetime of its excited state, and/or re-emission of fluorescent light at the longer wavelengths (lower energies) characteristic of the acceptor. When the fluorescent proteins physically separate, FRET effects are diminished or eliminated. (U.S. Patent No. 5,981,200).

For example, a cyan fluorescent protein is excited by light at roughly 425 - 450 nm wavelength and emits light in the range of 450 - 500 nm. Yellow fluorescent protein is excited by light at roughly 500 - 525 nm and emits light at 525 - 500 nm. If these two proteins are placed in solution, the cyan and yellow fluorescence may be separately visualized. However, if these two proteins are forced into close proximity with each other, the fluorescent properties will be altered by FRET. The bluish light emitted by CFP will be absorbed by YFP and re-emitted as yellow light. This means that when the proteins are stimulated with light at wavelength 450 nm, the cyan emitted light is greatly reduced and the yellow light, which is not normally stimulated at this wavelength, is greatly increased. FRET is typically monitored by measuring the spectrum of emitted light in response to stimulation with light in the excitation range of the donor and calculating a ratio between the donor-emitted light and the acceptor-emitted light. When the donor:acceptor emission ratio is high, FRET is not occurring and the two fluorescent proteins are not in close proximity. When the donor: acceptor emission ratio is low, FRET is occurring and the two fluorescent proteins are in close proximity. In this manner, the interaction between a first and second polypeptide may be measured.

The occurrence of FRET also causes the fluorescence lifetime of the donor fluorescent moiety to decrease. This change in fluorescence lifetime can be measured using a technique termed fluorescence lifetime imaging technology

(FLIM) (Verveer et al. (2000) *Science* 290: 1567-1570; Squire et al. (1999) *J. Microsc.* 193: 36; Verveer et al. (2000) *Biophys. J.* 78: 2127). Global analysis techniques for analyzing FLIM data have been developed. These algorithms use the understanding that the donor fluorescent moiety exists in only a limited number of states each with a distinct fluorescence lifetime. Quantitative maps of each state can
5 be generated on a pixel-by-pixel basis.

To perform FRET-based assays, the POSH polypeptide and the interacting protein of interest are both fluorescently labeled. Suitable fluorescent labels are, in view of this specification, well known in the art. Examples are provided below, but
10 suitable fluorescent labels not specifically discussed are also available to those of skill in the art. Fluorescent labeling may be accomplished by expressing a polypeptide as a fusion protein with a fluorescent protein, for example fluorescent proteins isolated from jellyfish, corals and other coelenterates. Exemplary fluorescent proteins include the many variants of the green fluorescent protein (GFP)
15 of *Aequoria victoria*. Variants may be brighter, dimmer, or have different excitation and/or emission spectra. Certain variants are altered such that they no longer appear green, and may appear blue, cyan, yellow or red (termed BFP, CFP, YFP and RFP, respectively). Fluorescent proteins may be stably attached to polypeptides through a variety of covalent and noncovalent linkages, including, for example, peptide bonds
20 (eg. expression as a fusion protein), chemical cross-linking and biotin-streptavidin coupling. For examples of fluorescent proteins, see U.S. Patents 5,625,048; 5,777,079; 6,066,476; 6,124,128; Prasher et al. (1992) *Gene*, 111:229-233; Heim et al. (1994) *Proc. Natl. Acad. Sci., USA*, 91:12501-04; Ward et al. (1982) *Photochem. Photobiol.*, 35:803-808 ; Levine et al. (1982) *Comp. Biochem. Physiol.*, 72B:77-85;
25 Tersikh et al. (2000) *Science* 290: 1585-88.

Other exemplary fluorescent moieties well known in the art include derivatives of fluorescein, benzoxadioazole, coumarin, eosin, Lucifer Yellow, pyridyloxazole and rhodamine. These and many other exemplary fluorescent moieties may be found in the *Handbook of Fluorescent Probes and Research*
30 *Chemicals* (2000, Molecular Probes, Inc.), along with methodologies for modifying polypeptides with such moieties. Exemplary proteins that fluoresce when combined with a fluorescent moiety include, yellow fluorescent protein from *Vibrio fischeri*

(Baldwin et al. (1990) *Biochemistry* 29:5509-15), peridinin-chlorophyll a binding protein from the dinoflagellate *Symbiodinium* sp. (Morris et al. (1994) *Plant Molecular Biology* 24:673:77) and phycobiliproteins from marine cyanobacteria such as *Synechococcus*, e.g., phycoerythrin and phycocyanin (Wilbanks et al. (1993) *J. Biol. Chem.* 268:1226-35). These proteins require flavins, peridinin-chlorophyll a and various phycobilins, respectively, as fluorescent co-factors.

FRET-based assays may be used in cell-based assays and in cell-free assays. FRET-based assays are amenable to high-throughput screening methods including Fluorescence Activated Cell Sorting and fluorescent scanning of microtiter arrays.

10 In a further embodiment, transcript levels may be measured in cells having higher or lower levels of POSH activity in order to identify genes that are regulated by POSH. Promoter regions for such genes (or larger portions of such genes) may be operatively linked to a reporter gene and used in a reporter gene-based assay to detect agents that enhance or diminish POSH-regulated gene expression. Transcript
15 levels may be determined in any way known in the art, such as, for example, Northern blotting, RT-PCR, microarray, etc. Increased POSH activity may be achieved, for example, by introducing a strong POSH expression vector. Decreased POSH activity may be achieved, for example, by RNAi, antisense, ribozyme, gene knockout, etc.

20 In general, where the screening assay is a binding assay (whether protein-protein binding, agent-protein binding, etc.), one or more of the molecules may be joined to a label, where the label can directly or indirectly provide a detectable signal. Various labels include radioisotopes, fluorescers, chemilumescers, enzymes, specific binding molecules, particles, e.g. magnetic particles, and the like.
25 Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin etc. For the specific binding members, the complementary member would normally be labeled with a molecule that provides for detection, in accordance with known procedures.

In further embodiments, the invention provides methods for identifying
30 targets for therapeutic intervention. A polypeptide that interacts with POSH or participates in a POSH-mediated process (such as cellular proliferation) may be used to identify candidate therapeutics. Such targets may be identified by identifying

proteins that associated with POSH (POSH-APs) by, for example, immunoprecipitation with an anti-POSH antibody, in silico analysis of high-throughput binding data, two-hybrid screens, and other protein-protein interaction assays described herein or otherwise known in the art in view of this disclosure.

5 Agents that bind to such targets or disrupt protein-protein interactions thereof, or inhibit a biochemical activity thereof may be used in such an assay. Targets that may be identified by such approaches include: a GTPase (eg. Rac, Rac1, Rho, Ras); an E2 enzyme, a cullin; a clathrin; AP-1; AP-2; an HSP70; an HSP90, Brca1, Bard1, Nef, PAK1, PAK2, PAK family, Vav, Cdc42, PI3K (e.g. p85 or p110), Nedd4, src

10 (src family), Tsg101, VASP, RNB6, WASP, N-WASP, a Gag; and KIAA0674, Similar to Spred-2, as well as, in certain embodiments, proteins known to be associated with clathrin-coated vesicles, proteins involved in the protein sorting pathway and proteins involved in a Rac signaling pathway.

A variety of other reagents may be included in the screening assay. These

15 include reagents like salts, neutral proteins, e.g. albumin, detergents, etc that are used to facilitate optimal protein-protein binding and/or reduce nonspecific or background interactions. Reagents that improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti- microbial agents, etc. may be used. The mixture of components are added in any order that provides for the requisite

20 binding. Incubations are performed at any suitable temperature, typically between 4° and 40° C. Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high-throughput screening.

In certain embodiments, a test agent may be assessed for its ability to perturb the localization of a POSH polypeptide, e.g. preventing POSH localization to the

25 nucleus and/ or the Golgi network.

10. Methods and Compositions for Treatment of Neoplastic Conditions

In a further aspect, the invention provides methods and compositions for treatment of neoplastic conditions, including benign tumors and malignant tumors,

30 i.e., cancers. Preferred therapeutics of the invention function by disrupting a biological activity of a POSH polypeptide or POSH complex.

Exemplary therapeutics of the invention include nucleic acid therapies such as for example RNAi constructs, antisense oligonucleotides, ribozyme, and DNA enzymes. Other POSH therapeutics include polypeptides, peptidomimetics, antibodies and small molecules.

5 Antisense therapies of the invention include methods of introducing antisense nucleic acids to disrupt the expression of POSH polypeptides or proteins that are necessary for POSH function.

 RNAi therapies include methods of introducing RNAi constructs to downregulate the expression of POSH polypeptides or proteins that are necessary for
10 POSH function. Exemplary RNAi therapeutics include any one of SEQ ID Nos: 15, 16, 18, 19, 21, 22, 24 and 25.

 Therapeutic polypeptides may be generated by designing polypeptides to mimic certain protein domains important in the formation of POSH complexes, such as, for example SH3 or RING domains. For example, a polypeptide comprising a
15 POSH SH3 domain such as for example the SH3 domain as set forth in SEQ ID No: 30 will compete for binding to a POSH SH3 domain and will therefore act to disrupt binding of a partner protein. In one embodiment, a binding partner may be Gag. In another embodiment, a binding partner may be Rac. In a further embodiment, a polypeptide that resembles an L domain may disrupt recruitment of Gag to the
20 POSH complex.

 In view of the specification, methods for generating antibodies directed to epitopes of POSH and POSH-interacting proteins are known in the art. Antibodies may be introduced into cells by a variety of methods. One exemplary method comprises generating a nucleic acid encoding a single chain antibody that is capable
25 of disrupting a POSH complex. Such a nucleic acid may be conjugated to antibody that binds to receptors on the surface of target cells. It is contemplated that in certain embodiments, the antibody may target viral proteins that are present on the surface of infected cells, and in this way deliver the nucleic acid only to infected cells. Once bound to the target cell surface, the antibody is taken up by endocytosis;
30 and the conjugated nucleic acid is transcribed and translated to produce a single chain antibody that interacts with and disrupts the targeted POSH complex. Nucleic acids expressing the desired single chain antibody may also be introduced into cells

using a variety of more conventional techniques, such as viral transfection (eg. using an adenoviral system) or liposome-mediated transfection.

Small molecules of the invention may be identified for their ability to modulate the formation of POSH complexes, as described above.

5 In view of the teachings herein, one of skill in the art will understand that the methods and compositions of the invention are applicable to a wide range of neoplastic conditions, including benign growth such as warts, precancerous growth, benign tumors and malignant tumors, i.e. cancers. While not intended to be limiting, the present invention is applicable for treating thyroid carcinoma, liver cancer
10 (hepatocellular cancer), lung cancer, cervical cancer, ovarian cancer, renal cell carcinoma, lymphoma, osteosarcoma, liposarcoma, leukemia, breast carcinoma, breast adeno-carcinoma, papillomas (e.g. HPV-related papillomas, condylomata acuminatum), cervical squamous intraepithelial lesions, oral cancer, penile cancer, clear cell sarcoma, etc.

15

11. Methods and compositions for diagnosis of neoplastic conditions

In general, a neoplastic condition such as a cancer may be detected in a patient based on the presence of one or more tumor proteins and/or polynucleotides encoding such proteins in a biological sample (for example, blood, sera, urine and/or
20 tumor biopsies) obtained from the patient. In other words, such proteins may be used as markers to indicate the presence or absence of a cancer. Given that POSH is differentially expressed in certain cancer tissues versus normal healthy tissues, the expression level of POSH in a biological sample may be used to indicate the presence or absence of a cancer.

25 Disclosed herein are methods and compositions to detect POSH expression level in biological samples from a subject. Binding agents that specifically bind to POSH mRNA or polypeptide may be used to detect POSH. For example, Polynucleotide primers and probes may be used to detect the level of mRNA encoding POSH. For another example, antibodies, or antigen-binding fragment
30 thereof, may be used to detect the level of POSH polypeptide. There are a variety of assay formats known to those of ordinary skill in the art for using a binding agent to detect polypeptide markers in a sample. See, e.g., Harlow and Lane, Antibodies: A

Laboratory Manual, Cold Spring Harbor Laboratory, 1988. In general, the presence or absence of a cancer in a patient may be determined by (a) contacting a biological sample obtained from a patient with a binding agent; (b) detecting in the sample a level of polypeptide that binds to the binding agent; and (c) comparing the level of polypeptide in the patient with that in a healthy normal control subject.

In an embodiment, the assay involves the use of binding agent immobilized on a solid support to bind to and remove the polypeptide from the remainder of the sample. The bound polypeptide may then be detected using a detection reagent that contains a reporter group and specifically binds to the binding agent/polypeptide complex. Such detection reagents may comprise, for example, a binding agent that specifically binds to the polypeptide or an antibody or other agent that specifically binds to the binding agent, such as an anti-immunoglobulin, protein G, protein A or a lectin. Alternatively, a competitive assay may be utilized, in which a polypeptide is labeled with a reporter group and allowed to bind to the immobilized binding agent after incubation of the binding agent with the sample. The extent to which components of the sample inhibit the binding of the labeled polypeptide to the binding agent is indicative of the reactivity of the sample with the immobilized binding agent. Suitable polypeptides for use within such assays include full length POSH proteins and portions thereof to which the binding agent binds, as described above.

The solid support may be any material known to those of ordinary skill in the art to which the tumor protein may be attached. For example, the solid support may be a test well in a microtiter plate or a nitrocellulose or other suitable membrane. Alternatively, the support may be a bead or disc, such as glass, fiberglass, latex or a plastic material such as polystyrene or polyvinylchloride. The support may also be a magnetic particle or a fiber optic sensor, such as those disclosed, for example, in U.S. Pat. No. 5,359,681. The binding agent may be immobilized on the solid support using a variety of techniques known to those of skill in the art, which are amply described in the patent and scientific literature. In the context of the present invention, the term "immobilization" refers to both noncovalent association, such as adsorption, and covalent attachment (which may be a direct linkage between the agent and functional groups on the support or may be a linkage by way of a cross-

linking agent). Immobilization by adsorption to a well in a microtiter plate or to a membrane is preferred. In such cases, adsorption may be achieved by contacting the binding agent, in a suitable buffer, with the solid support for a suitable amount of time. The contact time varies with temperature, but is typically between about 1 hour
5 and about 1 day. In general, contacting a well of a plastic microtiter plate (such as polystyrene or polyvinylchloride) with an amount of binding agent ranging from about 10 ng to about 10 μ g, and preferably about 100 ng to about 1 μ g, is sufficient to immobilize an adequate amount of binding agent.

Covalent attachment of binding agent to a solid support may generally be
10 achieved by first reacting the support with a bifunctional reagent that will react with both the support and a functional group, such as a hydroxyl or amino group, on the binding agent. For example, the binding agent may be covalently attached to supports having an appropriate polymer coating using benzoquinone or by
condensation of an aldehyde group on the support with an amine and an active
15 hydrogen on the binding partner (see, e.g., Pierce Immunotechnology Catalog and Handbook, 1991, at A12-A13).

In certain embodiments, the assay is a two-antibody sandwich assay. This assay may be performed by first contacting an antibody that has been immobilized on a solid support, commonly the well of a microtiter plate, with the sample, such
20 that polypeptides within the sample are allowed to bind to the immobilized antibody. Unbound sample is then removed from the immobilized polypeptide-antibody complexes and a detection reagent (preferably a second antibody capable of binding to a different site on the polypeptide) containing a reporter group is added. The amount of detection reagent that remains bound to the solid support is then
25 determined using a method appropriate for the specific reporter group.

More specifically, once the antibody is immobilized on the support as described above, the remaining protein binding sites on the support are typically blocked. Any suitable blocking agent known to those of ordinary skill in the art, such as bovine serum albumin or Tween 20.TM. (Sigma Chemical Co., St. Louis,
30 Mo.). The immobilized antibody is then incubated with the sample, and polypeptide is allowed to bind to the antibody. The sample may be diluted with a suitable diluent, such as phosphate-buffered saline (PBS) prior to incubation. In general, an

appropriate contact time (i.e., incubation time) is a period of time that is sufficient to detect the presence of polypeptide within a sample obtained from an individual with lung cancer. Preferably, the contact time is sufficient to achieve a level of binding that is at least about 95% of that achieved at equilibrium between bound and unbound polypeptide. Those of ordinary skill in the art will recognize that the time necessary to achieve equilibrium may be readily determined by assaying the level of binding that occurs over a period of time. At room temperature, an incubation time of about 30 minutes is generally sufficient. Unbound sample may then be removed by washing the solid support with an appropriate buffer, such as PBS containing 0.1% Tween 20.TM.. The second antibody, which contains a reporter group, may then be added to the solid support. Preferred reporter groups include those groups recited above.

The detection reagent is then incubated with the immobilized antibody-polypeptide complex for an amount of time sufficient to detect the bound polypeptide. An appropriate amount of time may generally be determined by assaying the level of binding that occurs over a period of time. Unbound detection reagent is then removed and bound detection reagent is detected using the reporter group. The method employed for detecting the reporter group depends upon the nature of the reporter group. For radioactive groups, scintillation counting or autoradiographic methods are generally appropriate. Spectroscopic methods may be used to detect dyes, luminescent groups and fluorescent groups. Biotin may be detected using avidin, coupled to a different reporter group (commonly a radioactive or fluorescent group or an enzyme). Enzyme reporter groups may generally be detected by the addition of substrate (generally for a specific period of time), followed by spectroscopic or other analysis of the reaction products.

In a related embodiment, the assay is performed in a flow-through or strip test format, wherein the binding agent is immobilized on a membrane, such as nitrocellulose. In the flow-through test, polypeptides within the sample bind to the immobilized binding agent as the sample passes through the membrane. A second, labeled binding agent then binds to the binding agent-polypeptide complex as a solution containing the second binding agent flows through the membrane. The detection of bound second binding agent may then be performed as described above.

In the strip test format, one end of the membrane to which binding agent is bound is immersed in a solution containing the sample. The sample migrates along the membrane through a region containing second binding agent and to the area of immobilized binding agent. Concentration of second binding agent at the area of immobilized antibody indicates the presence of a cancer. Typically, the concentration of second binding agent at that site generates a pattern, such as a line, that can be read visually. The absence of such a pattern indicates a negative result. In general, the amount of binding agent immobilized on the membrane is selected to generate a visually discernible pattern when the biological sample contains a level of polypeptide that would be sufficient to generate a positive signal in the two-antibody sandwich assay, in the format discussed above. Preferred binding agents for use in such assays are antibodies and antigen-binding fragments thereof. Preferably, the amount of antibody immobilized on the membrane ranges from about 25 ng to about 1 .mu.g, and more preferably from about 50 ng to about 500 ng. Such tests can typically be performed with a very small amount of biological sample.

Of course, numerous other assay protocols exist that are suitable for use with the tumor proteins or binding agents of the present invention. The above descriptions are intended to be exemplary only. For example, it will be apparent to those of ordinary skill in the art that the above protocols may be readily modified to use lung tumor polypeptides to detect antibodies that bind to such polypeptides in a biological sample. The detection of such lung tumor protein specific antibodies may correlate with the presence of a cancer.

As noted above, a cancer may also, or alternatively, be detected based on the level of mRNA encoding POSH protein in a biological sample. For example, at least two oligonucleotide primers may be employed in a polymerase chain reaction (PCR) based assay to amplify a portion of POSH cDNA derived from a biological sample, wherein at least one of the oligonucleotide primers is specific for (i.e., hybridizes to) a polynucleotide encoding the POSH protein. The amplified cDNA is then separated and detected using techniques well known in the art, such as gel electrophoresis. Similarly, oligonucleotide probes that specifically hybridize to a polynucleotide encoding POSH protein may be used in a hybridization assay to detect the presence of polynucleotide encoding the POSH protein in a biological sample.

To permit hybridization under assay conditions, oligonucleotide primers and probes should comprise an oligonucleotide sequence that has at least about 60%, preferably at least about 75% and more preferably at least about 90%, identity to a portion of a polynucleotide encoding a lung tumor protein that is at least 10
5 nucleotides, and preferably at least 20 nucleotides, in length. Preferably, oligonucleotide primers and/or probes will hybridize to a polynucleotide encoding a polypeptide disclosed herein under moderately stringent conditions, as defined above. Oligonucleotide primers and/or probes which may be usefully employed in the diagnostic methods described herein preferably are at least 10-40 nucleotides in
10 length. Techniques for both PCR based assays and hybridization assays are well known in the art (see, for example, Mullis et al., Cold Spring Harbor Symp. Quant. Biol., 51:263, 1987; Erlich ed., PCR Technology, Stockton Press, NY, 1989).

One preferred assay employs RT-PCR, in which PCR is applied in conjunction with reverse transcription. Typically, RNA is extracted from a
15 biological sample, such as biopsy tissue, and is reverse transcribed to produce cDNA molecules. PCR amplification using at least one specific primer generates a cDNA molecule, which may be separated and visualized using, for example, gel electrophoresis. Amplification may be performed on biological samples taken from a test patient and from an individual who is not afflicted with a cancer. The
20 amplification reaction may be performed on several dilutions of cDNA spanning two orders of magnitude. A two-fold or greater increase in expression in several dilutions of the test patient sample as compared to the same dilutions of the non-cancerous sample is typically considered positive.

In another embodiment, the disclosed compositions may be used as markers
25 for the progression of cancer. In this embodiment, assays as described above for the diagnosis of a cancer may be performed over time, and the change in the level of reactive polypeptide(s) or polynucleotide evaluated. For example, the assays may be performed every 24-72 hours for a period of 6 months to 1 year, and thereafter performed as needed. In general, a cancer is progressing in those patients in whom
30 the level of polypeptide or polynucleotide detected increases over time. In contrast, the cancer is not progressing when the level of reactive polypeptide or polynucleotide either remains constant or decreases with time.

Certain in vivo diagnostic assays may be performed directly on a tumor. One such assay involves contacting tumor cells with a binding agent. The bound binding agent may then be detected directly or indirectly via a reporter group. Such binding agents may also be used in histological applications. Alternatively, polynucleotide probes may be used within such applications.

As noted above, to improve sensitivity, multiple tumor protein markers may be assayed within a given sample. It will be apparent that binding agents specific for POSH protein provided herein may be combined within a single assay. Further, multiple primers or probes may be used concurrently. The selection of tumor protein markers may be based on routine experiments to determine combinations that results in optimal sensitivity. In addition, or alternatively, assays for tumor proteins provided herein may be combined with assays for other known tumor antigens.

The present invention further provides kits for use within any of the above diagnostic methods. Such kits typically comprise two or more components necessary for performing a diagnostic assay. Components may be compounds, reagents, containers and/or equipment. For example, one container within a kit may contain a monoclonal antibody or fragment thereof that specifically binds to POSH protein. Such antibodies or fragments may be provided attached to a support material, as described above. One or more additional containers may enclose elements, such as reagents or buffers, to be used in the assay. Such kits may also, or alternatively, contain a detection reagent as described above that contains a reporter group suitable for direct or indirect detection of antibody binding.

Alternatively, a kit may be designed to detect the level of mRNA encoding POSH protein in a biological sample. Such kits generally comprise at least one oligonucleotide probe or primer, as described above, that hybridizes to a polynucleotide encoding POSH protein. Such an oligonucleotide may be used, for example, within a PCR or hybridization assay. Additional components that may be present within such kits include a second oligonucleotide and/or a diagnostic reagent or container to facilitate the detection of a polynucleotide encoding POSH protein.

30

12. Effective Dose

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining The Ld50 (The Dose Lethal To 50% Of The Population) And The Ed50 (the dose therapeutically effective in 50% of the population). The dose ratio
5 between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic induces are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and,
10 thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the
15 dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (i.e., the concentration of the test compound which achieves a half-maximal
20 inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

13. Formulation and Use

25 Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients. Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by, for example, injection, inhalation or insufflation (either through the mouth or the
30 nose) or oral, buccal, parenteral or rectal administration.

An exemplary composition of the invention comprises an RNAi mixed with a delivery system, such as a liposome system, and optionally including an acceptable

excipient. In a preferred embodiment, the composition is formulated for topical administration for, e.g. herpes virus infections.

For such therapy, the compounds of the invention can be formulated for a variety of loads of administration, including systemic and topical or localized
5 administration. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, Meade Publishing Co., Easton, PA. For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the compounds of the invention can be formulated in liquid solutions, preferably in physiologically compatible
10 buffers such as Hank's solution or Ringer's solution. In addition, the compounds may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with
15 pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets may be
20 coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending
25 agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., ationd oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

30 Preparations for oral administration may be suitably formulated to give controlled release of the active compound. For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional

manner. For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, 5 dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g., gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

10 The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory 15 agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases 20 such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with 25 suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier 30 to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid derivatives. In addition, detergents may be used to facilitate permeation.

Transmucosal administration may be through nasal sprays or using suppositories. For topical administration, the oligomers of the invention are formulated into ointments, salves, gels, or creams as generally known in the art. A wash solution can be used locally to treat an injury or inflammation to accelerate healing.

5 The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

10 For therapies involving the administration of nucleic acids, the oligomers of the invention can be formulated for a variety of modes of administration, including systemic and topical or localized administration. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, Meade Publishing Co., Easton, PA. For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, intranodal, and subcutaneous
15 for injection, the oligomers of the invention can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the oligomers may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included.

20 Systemic administration can also be by transmucosal or transdermal means, or the compounds can be administered orally. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid derivatives. In
25 addition, detergents may be used to facilitate permeation. Transmucosal administration may be through nasal sprays or using suppositories. For oral administration, the oligomers are formulated into conventional oral administration forms such as capsules, tablets, and tonics. For topical administration, the oligomers of the invention are formulated into ointments, salves, gels, or creams as generally
30 known in the art.

EXEMPLIFICATION

The invention now being generally described, it will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

5

EXAMPLES

Example 1. Knockdown of POSH mRNA by siRNA duplexes

1. Objective:

Use RNAi to inhibit POSH gene expression and compare the efficiency of viral budding and GAG expression and processing in treated and untreated cells.

10

2. Study Plan:

HeLa SS-6 cells are transfected with mRNA-specific RNAi in order to knockdown the target proteins. Since maximal reduction of target protein by RNAi is achieved after 48 hours, cells are transfected twice – first to reduce target mRNAs, and subsequently to express the viral Gag protein. The second transfection is performed with pNLenv (plasmid that encodes HIV) and with low amounts of RNAi to maintain the knockdown of target protein during the time of gag expression and budding of VLPs. Reduction in mRNA levels due to RNAi effect is verified by RT-PCR amplification of target mRNA.

15

20 3. Methods, Materials, Solutions

a. Methods

i. Transfections according to manufacturer's protocol and as described in procedure.

ii. Protein determined by Bradford assay.

25 iii. SDS-PAGE in Hoeffer miniVE electrophoresis system. Transfer in Bio-Rad mini-protean II wet transfer system. Blots visualized using Typhoon system, and ImageQuant software (ABbiotech)

b. Materials

Material	Manufacturer	Catalog #	Batch #
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Lipofectamine 2000 (LF2000)	Life Technologies	11668-019	1112496
OptiMEM	Life Technologies	31985-047	3063119
RNAi Lamin A/C	Self	13	
RNAi TSG101 688	Self	65	
RNAi Posh 524	Self	81	
plenv11 PTAP	Self	148	
plenv11 ATAP	Self	149	
Anti-p24 polyclonal antibody	Seramun		A-0236/5-10-01
Anti-Rabbit Cy5 conjugated antibody	Jackson	144-175-115	48715
10% acrylamide Tris-Glycine SDS-PAGE gel	Life Technologies	NP0321	1081371
Nitrocellulose membrane	Schleicher & Schuell	401353	BA-83
NuPAGE 20X transfer buffer	Life Technologies	NP0006-1	224365
0.45µm filter	Schleicher & Schuell	10462100	CS1018-1

c. Solutions

Lysis Buffer	Compound	Concentration
	Tris-HCl pH 7.6	50mM
	MgCl ₂	15mM
	NaCl	150mM
	Glycerol	10%
	EDTA	1mM
	EGTA	1mM

	ASB-14 (add immediately before use)	1%
6X Sample Buffer	Tris-HCl, pH=6.8	1M
	Glycerol	30%
	SDS	10%
	DTT	9.3%
	Bromophenol Blue	0.012%
TBS-T	Tris pH=7.6	20mM
	NaCl	137mM
	Tween-20	0.1%

4. Procedure

a. Schedule

Day				
1	2	3	4	5
Plate cells	Transfection I (RNAi only)	Passage cells (1:3)	Transfection II (RNAi and pNlenv) (12:00, PM)	Extract RNA for RT-PCR (post transfection)
			Extract RNA for RT-PCR (pre-transfection)	Harvest VLPs and cells

5 b. Day 1

Plate HeLa SS-6 cells in 6-well plates (35mm wells) at concentration of 5×10^5 cells/well.

c. Day 2

2 hours before transfection replace growth medium with 2 ml growth medium without antibiotics.

Transfection I:

Reaction	RNAi name	TAGDA#	Reactions	RNAi [nM]	RNAi	A	B
					[20 μ M]	OPTiMEM	LF2000 mix
					μ l	(μ l)	(μ l)
1	Lamin A/C	13	2	50	12.5	500	500
2	Lamin A/C	13	1	50	6.25	250	250
3	TSG101 688	65	2	20	5	500	500
5	Posh 524	81	2	50	12.5	500	500

Transfections:

Prepare LF2000 mix : 250 μ l OptiMEM + 5 μ l LF2000 for each reaction. Mix by inversion, 5 times. Incubate 5 minutes at room temperature.

- 5 Prepare RNA dilution in OptiMEM (Table 1, column A). Add LF2000 mix dropwise to diluted RNA (Table 1, column B). Mix by gentle vortex. Incubate at room temperature 25 minutes, covered with aluminum foil.

Add 500 μ l transfection mixture to cells dropwise and mix by rocking side to side.

- 10 Incubate overnight.

d. Day 3

Split 1:3 after 24 hours. (Plate 4 wells for each reaction, except reaction 2 which is plated into 3 wells.)

e. Day 4

- 15 2 hours pre-transfection replace medium with DMEM growth medium without antibiotics.

Transfection II

Reaction	RNAi name	TAGDA#	Plasmid Reactions					
				RNAi				
				Plasmid [20 μ M] for				
				Plasmid for 2.4 μ g	10nM	OPTiMEM	LF2000 mix	
				(μ g/ μ l)	(μ l)	(μ l)	(μ l)	(μ l)
1	Lamin A/C	13	PTAP	3	3.4	3.75	750	750
2	Lamin A/C	13	ATAP	3	2.5	3.75	750	750
3	TSG101 688	65	PTAP	3	3.4	3.75	750	750

5 Posh 524 81 PTAP 3 3.4 3.75 750 750

Prepare LF2000 mix: 250µl OptiMEM + 5 µl LF2000 for each reaction. Mix by inversion, 5 times. Incubate 5 minutes at room temperature.

Prepare RNA+DNA diluted in OptiMEM (Transfection II, A+B+C)

- 5 Add LF2000 mix (Transfection II, D) to diluted RNA+DNA dropwise, mix by gentle vortex, and incubate 1h while protected from light with aluminum foil. Add LF2000 and DNA+RNA to cells, 500µl/well, mix by gentle rocking and incubate overnight.

f. Day 5

- 10 Collect samples for VLP assay (approximately 24 hours post-transfection) by the following procedure (cells from one well from each sample is taken for RNA assay, by RT-PCR).

g. Cell Extracts

- 15 i. Pellet floating cells by centrifugation (5min, 3000rpm at 4°C), save supernatant (continue with supernatant immediately to step h), scrape remaining cells in the medium which remains in the well, add to the corresponding floating cell pellet and centrifuge for 5 minutes, 1800rpm at 4°C.
- 20 ii. Wash cell pellet twice with ice-cold PBS.
- 20 iii. Resuspend cell pellet in 100µl lysis buffer and incubate 20 minutes on ice.
- iv. Centrifuge at 14,000rpm for 15min. Transfer supernatant to a clean tube. This is the cell extract.
- 25 v. Prepare 10µl of cell extract samples for SDS-PAGE by adding SDS-PAGE sample buffer to 1X, and boiling for 10 minutes. Remove an aliquot of the remaining sample for protein determination to verify total initial starting material. Save remaining cell extract at -80 °C.
- h. Purification of VLPs from cell media
- 30 i. Filter the supernatant from step g through a 0.45m filter.
- ii. Centrifuge supernatant at 14,000rpm at 4°C for at least 2h.
- iii. Aspirate supernatant carefully.

- iv. Re-suspend VLP pellet in hot (100°C warmed for 10 min at least) 1X sample buffer.
- v. Boil samples for 10 minutes, 100°C.
- i. Western Blot analysis
 - 5 i. Run all samples from stages A and B on Tris-Glycine SDS-PAGE 10% (120V for 1.5h.).
 - ii. Transfer samples to nitrocellulose membrane (65V for 1.5h.).
 - iii. Stain membrane with ponceau S solution.
 - iv. Block with 10% low fat milk in TBS-T for 1h.
 - 10 v. Incubate with anti p24 rabbit 1:500 in TBS-T o/n.
 - vi. Wash 3 times with TBS-T for 7min each wash.
 - vii. Incubate with secondary antibody anti rabbit cy5 1:500 for 30min.
 - viii. Wash five times for 10min in TBS-T
 - ix. View in Typhoon gel imaging system (Molecular Dynamics/APBitech)
 - 15 for fluorescence signal.

Results are shown in Figure 11.

Example 2. Exemplary POSH RT-PCR primers and siRNA duplexes

RT-PCR primers

	Name	Position	Sequence
Sense primer	POSH=271	271	5' CTTGCCTTGCCAGCATAC 3' (SEQ ID NO:12)
Anti-sense primer	POSH=926c	926C	5' CTGCCAGCATTCTTCAG 3' (SEQ ID NO:13)

siRNA duplexes:

- siRNA No: 153
- siRNA Name: POSH-230
- Position in mRNA 426-446
- 25 Target sequence: 5' AACAGAGGCTTGGAACCTG 3' SEQ ID NO: 14
- siRNA sense strand: 5' dTdTcAGAGGCCUUGGAAACCUG 3' SEQ ID NO: 15
- siRNA anti-sense strand: 5'dTdTcAGGUUCCAAGGCCUCUG 3' SEQ ID NO: 16
- siRNA No: 155

	siRNA Name:	POSH-442	
	Position in mRNA	638-658	
	Target sequence:	5' AAAGAGCCTGGAGACCTTAAA 3'	SEQ ID NO: 17
	siRNA sense strand:	5' ddTdTAGAGCCUGGAGACCUUAAA 3'	SEQ ID NO: 18
5	siRNA anti-sense strand:	5' ddTdTUUUAAGGUCUCCAGGCUCU 3'	SEQ ID NO: 19
	siRNA No:	157	
	siRNA Name:	POSH-U111	
	Position in mRNA	2973-2993	
10	Target sequence:	5' AAGGATTGGTATGTGACTCTG 3'	SEQ ID NO: 20
	siRNA sense strand:	5' dTdTGGAUUGGUAUGUGACUCUG 3'	SEQ ID NO: 21
	siRNA anti-sense strand:	5' dTdTCAGAGUCACAUACCAAUCC 3'	SEQ ID NO: 22
	siRNA No:	159	
15	siRNA Name:	POSH-U410	
	Position in mRNA	3272-3292	
	Target sequence:	5' AAGCTGGATTATCTCCTGTTG 3'	SEQ ID NO: 23
	siRNA sense strand:	5' ddTdTGCUGGAUUAUCUCCUGUUG 3'	SEQ ID NO: 24
	siRNA anti-sense strand:	5' ddTdTCAACAGGAGAUAAUCCAGC 3'	SEQ ID NO: 25
20	siRNA No.:	187	
	siRNA Name:	POSH-control	
	Position in mRNA:	None. Reverse to #153	
	Target sequence:	5' AAGTCCAAAGGTTCCGGAGAC 3'	SEQ ID
25	NO: 36		

Example 3. POSH modulates HeLa cell growth

HeLa cells subject to siRNA knockdown of POSH expression showed a reversible decrease in cell growth.

30 Protocol:

Day 1: Plate two 6-well plates with HeLa SS6 cells (5×10^5) cells/well.

Day 2: Change medium to medium without antibiotics.

Day 3: Perform the same transfection as noted below.

Transfection: 10nM RNAi: A2=RNAi #153; A3=RNAi#187 (see Example 2 for
5 RNAi information)

Prepare mix of LF2000+OptiMEM: 3.25ml optimem with 65ul LF2000, incubate together for 5 min. Add to reactions as noted above. Incubate for 25 min. Add 500ul to the corresponding well.

Reaction name	No. of reactions	RNAi oligo #	RNAi (ul)	OptiMEM (ul)	Mix (LF2000+OptiMEM)
A2	4	153	10nM=5ul	1000	1000
A3	4	187	10nM=5ul	1000	1000

10 Day 4: Harvest one of each reaction for both protein extraction and RNA. (#153 day 4 and #187 day 4). For the other set of reactions: count and split each well into (5×10^5 cells) for transfection next day.

Day 5: Perform the same transfection as noted above.

Day 6: Harvest one plate for protein and RNA extraction and the other split to
15 (5×10^5 cells) and (2.5×10^5 cells)

Day 8: Harvest and count and to the (5×10^5 cells) plate from day six.

Day10: Harvest and count the (2.5×10^5 cells) plate from day 6.

Protein extract: for POSH expression evaluation

- 20 1. Wash cells twice with cold PBS on ice.
2. Add PBS and scrape cells off to eppendorf tubes.
3. Centrifuge at 40 deg. C at 1800 rpm for 5 min.

4. Add 100ul of RIPA Buffer + protease inhibitors (PI3K) (1:200)+ EDTA (1:100) to the remaining cells on plate.
 5. Incubate on ice for 15 minutes.
 6. Centrifuge for 10 min in 40 deg. C at maximum speed.
 - 5 7. Proteins (15ug/ lane) were loaded onto a 10% tris glycine gel, resolved by electrophoresis, transferred to nitrocellulose membrane and probed with anti POSH antibody for 1h (1:2000 dilution). Detected was by ECL.
 8. Evaluations of protein levels were performed using an ImageQuant analyzer.
- Results are shown in Figure 12.

10

Example 4. POSH protein expression profile

A. Expression profile of POSH in different cell lines.

Cells from various cell lines were lysed and cell lysates were separated by SDS-PAGE. POSH expression was analyzed by Western blot analysis using anti-
15 POSH.

B. Tissue-specific expression of POSH protein.

SDS-PAGE fractionated tissue blots (Genotech, cat no.TB54, TB37) of normal (lower blot) and tumorous (upper blot) tissues (75 µg protein each lane) were probed with rabbit polyclonal antibody 6564 (raised against POSH specific peptide)
20 and detected by Enhanced Chemiluminescence using anti-rabbit horseradish peroxidase (Amersham cat no. NA934V) and exposure to film (Kodak Biomax). Films were digitized by table-top scanning (UMAX) and Adobe Photoshop 5.5 graphics program.

Results are shown in Figure 13.

25

Example 5. POSH expression in thyroid carcinoma.

Various normal and tumor tissue sections were analyzed by immunohistochemistry for the presence of POSH. For screening of various normal and cancer samples, a human low-density normal tissue array (InnoGenex catalog #

TS4201-05) and a human low-density cancer tissue array (InnoGenex catalog # TS4204-05) were used.

1. Immunohistochemistry protocol for POSH

Reagent

5

TBS x 10 (10x25mM TBS pH 7.5)

160gr NaCl

10

60gr Trizma base

4gr KCl

Fill up to 2 liters final volume with ddH₂O

Adjust pH to 7.5 with concentrated HCl (ab. 32 ml)

Autoclave

15

TBST

Add 1 ml Tween-20 to 2 liters of TBSx1 buffer to a final concentration of 0.05%

20

EDTA 1 mM, pH 8

Stock solution x100

25

37.2 gr Disodium salt EDTA in 1000 ml H₂O Autoclave

Dilute 1:100 and adjust the pH to 8 just before using it.

Other reagents

30

1. 3% hydrogen peroxide

2. Endogenous Avidin/Biotin Blocking (Zymed 00-4303 kit)

3. CasBlock (Zymed 00-8120)

4. normal swine serum (Dako)

5. DAKO Envision Plus system, HRP (AEC) kit K4009 (Rabbit).

35

6. Hematoxyline (Pioneer Research Chemicals)

7. Hydromount (National Diagnostics HS-106)

Staining Protocol

1. Deparaffinization and rehydration with xylene and graded alcohol (hood is required).

Prepare 3 bathes with xylene, 2 with absolute ethanol, 2 with 96% ethanol and 2 with 70% ethanol and incubate as follows:

5 Xylene - 3min x 3)

Ethanol absolute - 2 min x 2

Ethanol 96% - 2 min x 2

Ethanol 70% - 2 min x 2

10 Tap water x 3 (can be done in the sink, take care not to wash with running water directly on slides)

2. Microwave pretreatment

Transfer slides to a microwavable plastic holder. Put the slides in a 1000 ml glass beaker containing 600 ml of 1mM EDTA, pH 8.0.

15 **Note:** When staining few slides, the holder should be filled with empty glass slides up to 20 slides in order to keep the same heating conditions in all experiments.

Heating protocol: use 60% power for 30 min (it takes about 10 min to bring to a boil and then 20 min with boiling).

Cooling: Keep microwave door half open for 5 min. Do not take beaker out of oven.

20 Take beaker out of oven, fill it up to 1000 ml with ddH₂O and wait 10 min.

Note: It is possible to stop staining here for up to overnight. Leave slides in the beaker.

3. Wash with TBS/T 3 min x 3 (first wash can be shorter) in 3 glass bathes.

4. Peroxidase blocking

Prepare fresh 200 ml of 3% hydrogen peroxide in methanol.

25 Incubate at room temperature for 10 minutes.

5. Wash with TBS/T 3 min x 3

Endogenous Avidin/Biotin Blocking (Zymed 00-4303 kit)

a. Place paper towel in slide box (for 100 slides) and absorb it with water.

b. Level the box.

30 c. Wipe excess TBST from each slide

d. Place slide on box (face up) – work with 3-5 slides at a time. Avoid letting them get dried.

e. Apply 2 drops (100 µl) of reagent A (Avidin solution) to the tissue

- f. Incubate 10 min at RT.
- g. Wash with TBS/T 3 min x 3
- h. Repeat steps c. to g. with reagent B (d-Biotin solution)
6. Block with blocking solution for 60 minutes. Use CasBlock (Zymed 00-8120)
- 5 containing 5% normal swine serum (Dako).
7. Wipe the slide from excess blocking solution (do not rinse with TBS/T as before)
8. Add Primary antibody diluted 1:400 in blocking solution, cover with cover slide and incubate overnight at 4°C.
- 10 9. Discard cover slide and wash with TBS/T 3 min x 3.
10. Secondary antibody
Use DAKO Envision Plus system, Peroxidase (AEC) kit:
 - a. Apply 2 drops of solution 2 (Labelled polymer) to the tissue; Apply 2 drops of solution B (biotinylated secondary antibody) to the tissue
 - 15 b. Incubate 30 min at RT.
 - c. Wash with TBS/T 3 min x 3
 - d. Wipe excess TBST from each slide
11. Staining with DAKO AEC Substrate kit
- 20 DAKO Envision Plus system, Peroxidase (AEC) kit
Apply 2 drops of solution 3 (AEC+ substrate-Chromogen) to the tissue
Incubate 5-30 min. Staining development should be controlled under microscope.
12. Wash several times in tap water
- 25 13. Counterstain with Hematoxyline (4 min)
14. Wash several times in tap water
15. Cover with 2-3 drops of Hydromount. Do not wipe the slide before adding the Hydromount
16. Cover with cover-slide and let dry at room temperature.
- 30 Results, as shown in Figure 19, demonstrate that POSH is differentially expressed in thyroid carcinoma versus normal tissue.

Example 6. POSH expression in tumors

Various tumor tissue sections were analyzed by immunohistochemistry for the presence of POSH. For screening of various tumor samples a human low-density cancer tissue array (InnoGenex catalog # TS4204-05) were used.

- 5 Paraffin embedded tissues were probed with anti-POSH antibodies. A secondary alkaline phosphatase conjugated anti-rabbit antibody was used to detect POSH-bound primary antibody. The presence of red color marks the detection of POSH in the tissue section.

- 10 Figure 20 shows the immunohistochemistry of human tumor tissue sections: A. Lymphoma B. osteosarcoma C. liposarcoma D. normal lung (left panel), lung carcinoma (right panel). As Figure 20 shows, POSH expression is detected in lymphoma, osteosarcoma, liposarcoma and lung carcinoma.

Example 7. In-vitro assay of Human POSH self-ubiquitination

- 15 Recombinant hPOSH was incubated with ATP in the presence of E1, E2 and ubiquitin as indicated in each lane. Following incubation at 37°C for 30 minutes, reactions were terminated by addition of SDS-PAGE sample buffer. The samples were subsequently resolved on a 10% polyacrylamide gel. The separated samples were then transferred to nitrocellulose and subjected to immunoblot analysis with an
20 anti ubiquitin polyclonal antibody. The position of migration of molecular weight markers is indicated on the right.

- Poly-Ub: Ub-hPOSH conjugates, detected as high molecular weight adducts only in reactions containing E1, E2 and ubiquitin. hPOSH-176 and hPOSH-178 are a short and a longer derivatives (respectively) of bacterially expressed hPOSH; C, control

- 25 E3
preliminary steps in high-throughput screen

Objective

1. Test Ub detection with in a Ub chain as function of an E3 (HRD1) and POSH auto-Ubiquitination.
30 2. Test Boston Biochem reagents.

Materials

1. E1 recombinant from baculovirus
2. E2 Ubch5c from bacteria
3. Ubiquitin
4. POSH #178 (1-361) gst fusion-purified but degraded
5. POSH # 176 (1-269) gst fusion-purified but degraded
6. hsHRD1 soluble ring containing region
5. Bufferx12 (Tris 7.6 40 mM, DTT 1mM, MgCl₂ 5mM, ATP 2uM)
6. Dilution buffer (Tris 7.6 40mM, DTT 1mM, ovalbumin 1ug/ul)

protocol

	0.1ug/ul	0.5ug/ul	5ug/ul	0.4ug/ul	2.5ug/u/	0.8ug/ul	
	E1	E2	Ub	176	178	Hrd1	Bx12
-E1 (E2+176)	-----	0.5	0.5	1	-----	-----	10
-E2 (E1+176)	1	-----	0.5	1	-----	-----	9.5
-ub (E1+E2+176)	1	0.5	-----	1	-----	-----	9.5
E1+E2+176+Ub	1	0.5	0.5	1		-----	9
-E1 (E2+178)	-----	0.5	0.5	-----	1	-----	10
-E2 (E1+178)	1	-----	0.5	-----	1	-----	9.5
-ub (E1+E2+178)	1	0.5	-----	-----	1	-----	9.5
E1+E2+178+Ub	1	0.5	0.5	-----	1	-----1	9
Hrd1, E1+E2+Ub	1	0.5	0.5	-----	-----	1	8.5

10

1. Incubate for 30 minutes at 37°C.
2. Run 12% SDS PAGE gel and transfer to nitrocellulose membrane
3. Incubate with anti-Ubiquitin antibody.

Results, shown in Figure 21, demonstrate that human POSH has
 15 ubiquitin ligase activity.

Example 8. Co-immunoprecipitation of hPOSH with myc-tagged activated (V12) and dominant-negative (N17) Rac1

Hela cells were transfected with combinations of myc-Rac1 V12 or N17 and
 20 hPOSHdelRING-V5. 24 hours after transfection (efficiency 80% as measured by GFP) cells were collected, washed with PBS, and swollen in hypotonic lysis buffer

(10mM HEPES pH=7.9, 15mM KCl, 0.1mM EDTA, 2mM MgCl₂, 1mM DTT, and protease inhibitors). Cells were lysed by 10 strokes with dounce homogenizer and centrifuged 3000xg for 10 minutes to give supernatant (Fraction 1) and nuclei. Nuclei were washed with Fraction 2 buffer (0.2% NP-40, 10mM HEPES pH=7.9, 5 40mM KCl, 5% glycerol) to remove peripheral proteins. Nuclei were spun-down and supernatant collected (Fraction 2). Nuclear proteins were eluted in Fraction 3 buffer (20mM HEPES pH=7.9, 0.42M KCl, 25% glycerol, 0.1mM EDTA, 2mM MgCl₂, 1mM DTT) by rotating 30 minutes in cold. Insoluble proteins were spun-down 14000xg and solubilized in Fraction 4 buffer (1% Fos-Choline 14, 50mM 10 HEPES pH=7.9, 150mM NaCl, 10% glycerol, 1mM EDTA, 1.5mM MgCl₂, 2mM DTT). Half of the total extract was pre-cleared against Protein A sepharose for 1.5 hours and used for IP with 1 µg anti-myc (9E10, Roche 1-667-149) and Protein A sepharose for 2 hours. Immune complexes were washed extensively, and eluted in SDS-PAGE sample buffer. Gels were run, and proteins electro-transferred to 15 nitrocellulose for immunoblot as in figure 23. Endogenous POSH and transfected hPOSHdelRING-V5 are precipitated as a complex with Myc-Rac1 V12/N17 Results, shown in Figure 22, demonstrate that POSH co-immunoprecipitates with Rac1.

20 Example 9. POSH is localized at two sites one at the nuclei and one at the golgi (C)). After HIV transfection recruitment of POSH to the golgi is enhanced(D)

HeLa and 293T cells were transfected with pNenv-1. 24 hours post transfection the cells were incubated with primary antibodies against POSH alongside with either anti-p24 Gag (B) or one of the organelle markers as follows: 25 BiP- endoplasmatic reticulum (data not shown), GM130- golgi, (C,D) nucleoporin- nuclei matrix (E, F) and histone H1- nucleus (data not shown). Two different florescence labled secondary antibodies were used. Specific signals were obtained by laser-scanning confocal microscopy, and individual signals were overlaid to assess their relative positions. As shown in figure 23, POSH (A) is localized in the 30 nucleus and partially colocalized with HIV-1 Gag following transfection outside the nucleus, presumably in the nuclear matrix (B).

INCORPORATION BY REFERENCE

All of the patents and publications cited herein are hereby incorporated by reference.

5

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

10

What Is Claimed:

1. A method for identifying an antiproliferative agent comprising: providing a POSH polypeptide and a test agent; and identifying a test agent that interacts with the POSH polypeptide.+
- 5 2. The method of claim 1, wherein the POSH polypeptide is selected from the group consisting of SEQ ID NOS: 2, 5, 7, 9 and 11 and fragments comprising at least 20 consecutive amino acids of any of SEQ ID Nos: 2, 5, 7, 9, and 11.
3. The method of claim 1, wherein the POSH polypeptide is expressed in a cell.
4. The method of claim 1, wherein the POSH polypeptide is a purified polypeptide.
- 10 5. The method of any of claims 2-4, wherein the POSH polypeptide comprises a domain selected from the group consisting of: an SH3 domain and a RING domain.
6. The method of claim 5, wherein the test agent binds to a domain selected from the group consisting of: an SH3 domain and a RING domain.
7. The method of claim 6, wherein the test agent is a polypeptide, an antibody, a
15 small molecule or a peptidomimetic.
8. A method for identifying an antiproliferative agent comprising: providing a polypeptide involved in a POSH-mediated cellular proliferation and a test agent; and identifying a test agent that interacts with the polypeptide involved in the POSH-mediated cellular proliferation.
- 20 9. The method of claim 8, wherein the polypeptide involved in a POSH-mediated cellular proliferation is selected from the group consisting of a polypeptide comprising a RING domain and a SH3 domain, Brca1, Rac, Rac1, Vav, Cdc42, and PI3K.
10. The method of claim 9, wherein the test agent is a polypeptide, an antibody, a
25 small molecule or a peptidomimetic.

11. A method for identifying an antiproliferative agent comprising: providing a POSH nucleic acid and a test agent; and identifying a test agent that binds to the POSH nucleic acid.
12. The method of claim 11, wherein the POSH nucleic acid is selected from the group consisting of SEQ ID Nos: 1, 3, 4, 6, 8, and 10.
13. The method of claim 11, wherein the test agent is a ribonucleic acid, an antisense oligonucleotide, a RNAi construct, a DNA enzyme, and a ribozyme.
14. The method of claim 11, wherein binding of the test agent to said POSH nucleic acid decreases the level of a POSH transcript.
15. A method for identifying an antiproliferative agent comprising: providing a nucleic acid involved in a POSH-mediated cellular proliferation and a test agent; and identifying a test agent that interacts with the nucleic acid involved in the POSH-mediated cellular proliferation.
16. The method of claim 15, wherein the nucleic acid involved in the POSH-mediated cellular proliferation is selected from the group consisting of a nucleic acid encoding polypeptide comprising a RING domain and a SH3 domain, Bcr1, Rac, Rac1, Vav, Cdc42, and PI3K.
17. The method of claim 16, wherein the test agent is a ribonucleic acid, an antisense oligonucleotide, a RNAi construct, a DNA enzyme, and a ribozyme.
18. A method for identifying an antiapoptotic agent comprising: providing a POSH polypeptide and a test agent, wherein the POSH polypeptide is at least 95% identical to SEQ ID NO:2; and identifying a test agent that binds to the POSH polypeptide.
19. The method of claim 18, wherein the test agent binds to a domain selected from the group consisting of: an SH3 domain and a RING domain.
20. The method of claim 18, wherein the POSH polypeptide is expressed in a cell.

21. The method of claim 18, wherein the POSH polypeptide is a purified polypeptide.
22. A method for identifying an antiapoptotic agent comprising identifying a test agent that binds to a POSH nucleic acid.
- 5 23. The method of claim 22, wherein the test agent is a ribonucleic acid.
24. The method of any of claims 18-23, further comprising: administering a composition comprising the test agent to a cell overexpressing a POSH polypeptide; and measuring the effect of the test agent on POSH-induced apoptosis.
25. A method for inhibiting cellular proliferation in a subject in need thereof,
10 comprising administering an effective amount of an agent that inhibits a POSH activity.
26. The method of claim 25, wherein the agent modulates the Ubiquitin ligase activity of the POSH polypeptide.
27. The method of claim 25, wherein the agent is selected from the group consisting
15 of a small molecule, an antibody, a fragment of an antibody, a peptidomimetic, and a polypeptide.
28. The method of claim 25, wherein the agent inhibits the interaction between a POSH polypeptide and a POSH-AP.
29. The method of claim 28, wherein the POSH-AP is selected from the group
20 consisting of: an E2, a POSH polypeptide, a ubiquitin, and a GTPase.
30. The method of claim 29, wherein the GTPase is Rac.
31. The method of claim 30, wherein the Rac is Rac1.
32. The method of claim 25, wherein the agent selected from the group consisting
25 of an antisense oligonucleotide, an RNAi construct, a DNA enzyme, and a ribozyme.

33. The method of claim 32, wherein the RNAi construct is selected from the group consisting of any one of SEQ ID Nos; 15, 16, 18, 19, 21, 22, 24, and 25.
34. The method of claim 32, wherein the agent decreases the level of POSH mRNA.
35. The method of claim 25, wherein the subject has a neoplastic condition.
- 5 36. The method of claim 35, wherein the neoplastic condition is cancer.
37. The method of claim 36, wherein the cancer is selected from a group consisting of: thyroid carcinoma, liver cancer (hepatocellular cancer), lung cancer, cervical cancer, ovarian cancer, renal cell carcinoma, lymphoma, osteosarcoma, liposarcoma, leukemia, breast carcinoma and breast adeno-carcinoma.
- 10 38. A non-human transgenic animal comprises a plurality of cells containing one or more recombinant constructs of a POSH gene, wherein expression of said POSH gene mitigates a POSH loss of function phenotype.
39. The non-human transgenic animal of claim 38, wherein said one or more recombinant constructs are introduced into said animal or an ancestor of said animal,
- 15 at an embryonic stage.
40. An isolated cell of the transgenic animal of claim 38.
41. The cell of claim 40, wherein the cell is a germ cell.
42. The cell of claim 40, wherein the cell is a somatic cell.
43. The non-human transgenic animal of claim 38, wherein all germ cells and
- 20 somatic cells contain one or more recombinant constructs of said POSH gene.
44. The non-human transgenic animal of claim 39, wherein said recombinant construct is introduced into the embryo by microinjection, electroporation, or lipofection.

45. The non-human transgenic animal of claim 38, wherein the non-human animal is selected from the group consisting of a mouse, rat, pig, sheep, goat, rabbit, cow, and a monkey.
46. The non-human animal of claim 38, wherein the POSH gene is human POSH transgene.
47. A method of constructing the non-human transgenic animal of claim 38, comprising introducing a POSH transgene, into a fertilized egg and implanting said fertilized egg.
48. The non-human transgenic animal of claim 38, wherein the POSH loss of function phenotype is a decrease in disease-associated cellular proliferation.
49. A method of screening for a substance to be used for treating a neoplastic condition, comprising administering a test compound to the non-human animal of claim 38, and assaying efficacy of said test compound in potentiating the POSH loss of function phenotype.
50. A method of evaluating the antiproliferation potential of an agent comprising (i) contacting transgenic non-human animal of step (i) with a test agent, and (ii) comparing the cellular proliferation in a sample from the treated animal with the cellular proliferation in a sample from an untreated transgenic animal or a transgenic animal treated with a control agent, wherein the difference in the cellular proliferation in the treated animal, relative to the cellular proliferation in the absence of treatment or treatment with a control agent, indicates the anti-proliferative potential of the test compound.
51. A method of evaluating an anti-proliferative activity of a test compound, comprising:
- (i) contacting the non-human transgenic animal or germline and somatic cells expressing a human POSH transgene, or a sample of cells derived therefrom with a test agent; and
 - (ii) determining the cellular proliferation in a specimen from the transgenic animal or in the sample of cells, wherein a statistically significant decrease in the cellular

proliferation, relative to the cellular proliferation in the absence of the test agent, indicates the test compound is a potential anti-proliferative agent.

52. A method of inhibiting cellular proliferation comprising inhibiting a ubiquitin-related activity of a POSH polypeptide.

- 5 53. The method of claim 52, wherein the POSH polypeptide is selected from the group consisting of SEQ ID NOS: 2, 5, 7, 9 and 11 and fragments comprising at least 20 consecutive amino acids of any of SEQ ID Nos: 2, 5, 7, 9, and 11.

54. The method of claim 52, comprising inhibiting an activity of the RING domain of the POSH polypeptide.

- 10 55. The method of claim 52, wherein the cellular proliferation is inhibited by administering an agent selected from the group consisting of a small molecule, an antibody, a peptidomimetic, and a polypeptide.

56. The method of claim 55, wherein the agent is an antibody or a fragment thereof, specifically immunoreactive with an epitope of SEQ ID No: 30.

- 15 57. The method of claim 52, wherein cellular proliferation is inhibited by administering an agent selected from the group consisting of a an antisense oligonucleotide, an RNAi construct, a DNA enzyme, and a ribozyme.

58. The method of claim 57, wherein the RNAi construct is selected from the group consisting of any one of SEQ ID Nos; 15, 16, 18, 19, 21, 22, 24, and 25.

- 20 59. A therapeutic composition for a neoplastic condition comprising an inhibitor of any one of a POSH polypeptide, a polypeptide set forth in SEQ ID No: 26, and a polypeptide set forth in SEQ ID No: 30, and a pharmaceutically acceptable excipient.

- 25 60. The therapeutic composition of claim 59, wherein the inhibitor is selected from the group consisting of a small molecule, an antibody, a polypeptide, and a peptidomimetic.

61. The therapeutic composition of claim 59, wherein the inhibitor disrupts the interaction between a POSH polypeptide and POSH-AP and/or inhibits a ubiquitin-related activity of a POSH polypeptide.
62. The therapeutic composition of claim 59, wherein the inhibitor is a polypeptide
5 of SEQ ID NO: 30.
63. The therapeutic composition of claim 59, wherein the inhibitor is selected from the group consisting of an antisense oligonucleotide, a DNA enzyme, a RNAi construct, and a ribozyme.
64. The therapeutic composition of claim 59, wherein the RNAi construct is
10 selected from the group consisting of any one of SEQ ID Nos; 15, 16, 18, 19, 21, 22, 24, and 25.
65. The therapeutic composition of claim 59, wherein the neoplastic condition is cancer.
66. The therapeutic composition of claim 65, wherein the cancer is selected from a
15 group consisting of: thyroid carcinoma, liver cancer (hepatocellular cancer), lung cancer, cervical cancer, ovarian cancer, renal cell carcinoma, lymphoma, osteosarcoma, liposarcoma, leukemia, breast carcinoma and breast adeno-carcinoma.
67. A method of inhibiting disease-associated cellular proliferation comprising administering an agent to a subject in need thereof wherein said agent inhibits
20 POSH-mediated cellular proliferation.
68. The method of claim 67, wherein the agent inhibits a polypeptide involved in the POSH-mediated cellular proliferation.
69. The method of claim 68, wherein the polypeptide involved in the POSH-mediated cellular proliferation is selected from the group consisting of a nucleic acid
25 encoding polypeptide comprising a RING domain and a SH3 domain, Brca1, Rac, Rac1, Vav, Cdc42, and PI3K.

70. A method of identifying targets for therapeutic intervention comprising identifying a polypeptide that associates with anyone of a POSH polypeptide or a polypeptide involved in a POSH-mediated cellular proliferation.

71. The method of claim 70, wherein the polypeptide involved in a POSH-mediated cellular proliferation is selected from the group consisting of a nucleic acid encoding polypeptide comprising a RING domain and a SH3 domain, Brca1, Rac, Rac1, Vav, Cdc42, and PI3K.

72. A method for evaluating the anti-proliferative potential of a compound comprising: forming a mixture comprising a ubiquitin; an E1; an E2; and a POSH polypeptide; adding a test agent; and detecting ubiquitin-ligase activity of said POSH polypeptide wherein a compound that modulates the ligase activity of said POSH polypeptide is a potential anti-proliferative agent.

73. A diagnostic method for detecting a neoplastic condition in a subject, said method comprising: (i) isolating a biological sample from a subject being tested, (ii) contacting the biological sample with a detecting agent to determine POSH expression level in the biological sample of the subject; and (iii) comparing the POSH expression level in the subject with the POSH expression level in a normal healthy control, wherein a statistically significant increase in the POSH expression level, relative to the POSH expression level in the control, indicates the presence of a neoplastic condition.

74. The diagnostic method of claim 73, wherein the POSH expression level is POSH polypeptide expression level.

75. The diagnostic method of claim 73, wherein the POSH expression level is POSH mRNA transcript expression level.

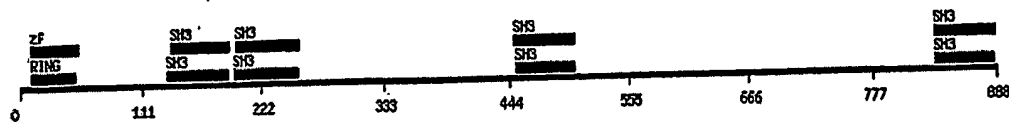
76. The diagnostic method of claim 73, wherein the detecting agent is an antibody which selectively binds to POSH protein.

77. The diagnostic method of claim 73, wherein the detecting agent is a nucleic acid which selectively binds to POSH mRNA.

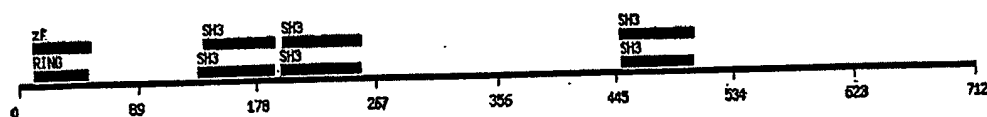
78. The diagnostic method of claim 73, wherein the neoplastic condition is a cancer.
79. The diagnostic method of claim 73, wherein the cancer is selected from a group consisting of: thyroid carcinoma, liver cancer (hepatocellular cancer), lung cancer, cervical cancer, ovarian cancer, renal cell carcinoma, lymphoma, osteosarcoma,
5 liposarcoma, leukemia, breast carcinoma and breast adeno-carcinoma.
80. A method for inhibiting disease associated cellular proliferation comprising administering a RNAi construct.
81. The method of claim 80, wherein the RNAi construct is selected from the group consisting of any one of SEQ ID Nos: 15, 16, 18, 19, 21, 22, 24, and 25.
- 10 82. The method of claim 81, wherein said disease associated cellular proliferation is a neoplastic condition.
83. The method of claim 82, wherein the neoplastic condition is cancer.
84. The method of claim 83, wherein the cancer is selected from a group consisting of: thyroid carcinoma, liver cancer (hepatocellular cancer), lung cancer, cervical
15 cancer, ovarian cancer, renal cell carcinoma, lymphoma, osteosarcoma, liposarcoma, leukemia, breast carcinoma and breast adeno-carcinoma.

Figure 18: POSH Domain Analysis

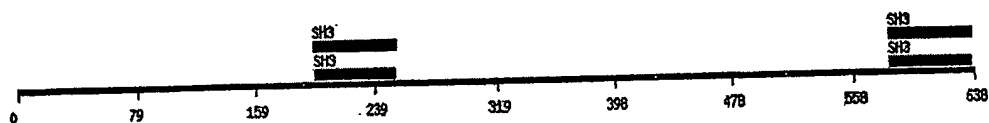
hPOSH protein sequence :



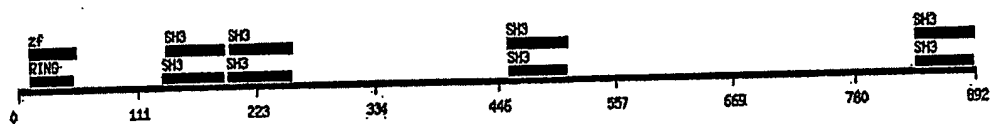
N terminus protein fragment of hPOSH (public gi:10432612):



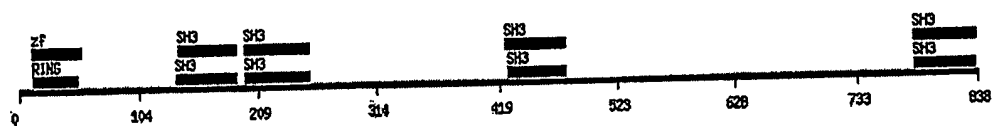
C terminus protein fragment of hPOSH (public gi:7959249):



Mouse POSH Protein sequence (Public gi: 10946922):



Drosophila melanogaster POSH protein sequence (public gi:17737481)



Thyroid carcinoma



Thyroid gland

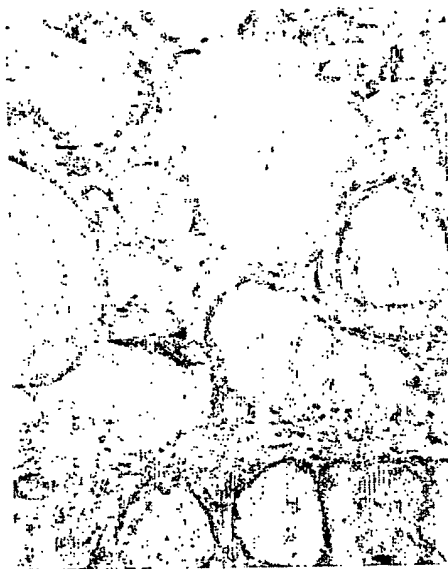
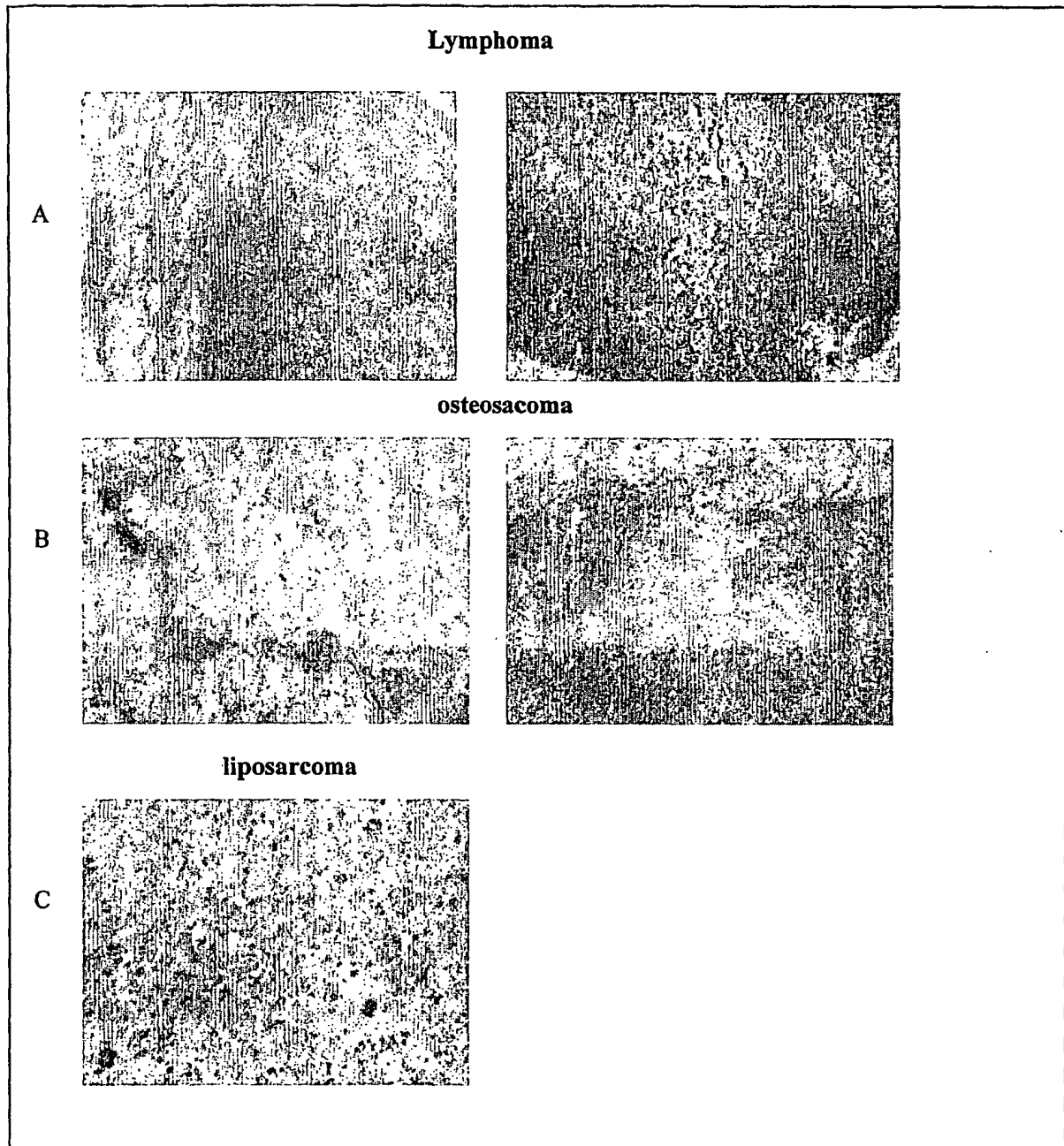


Figure 19. POSH expression in thyroid carcinoma

Figure 20. POSH expression in tumors



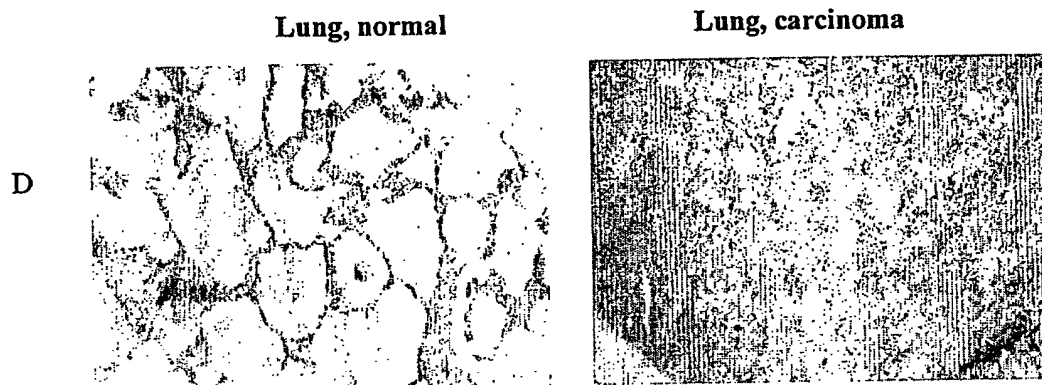
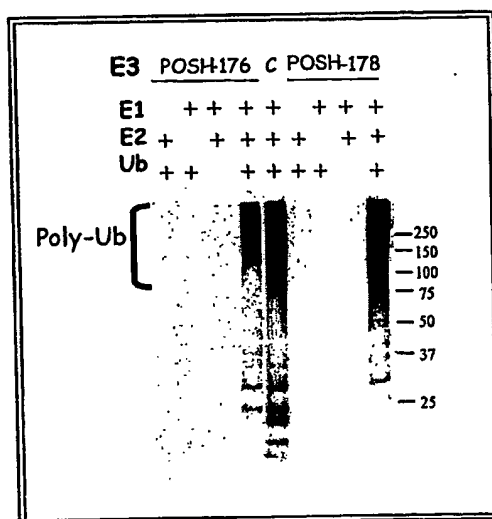


Figure 20. POSH expression in tumors

Figure 21: Human POSH has ubiquitin ligase activity



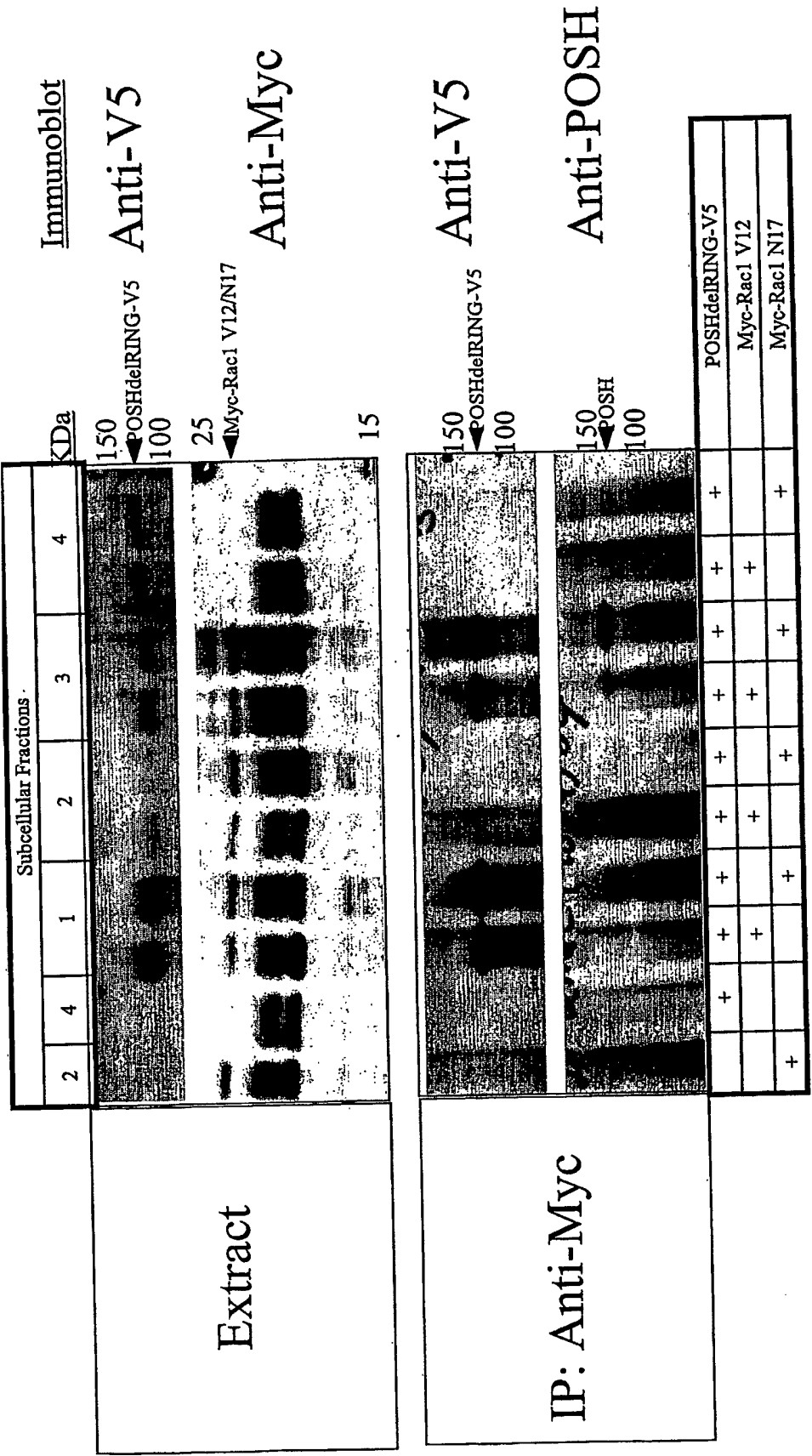


Figure 22. Human POSH co-immunoprecipitates with RAC1

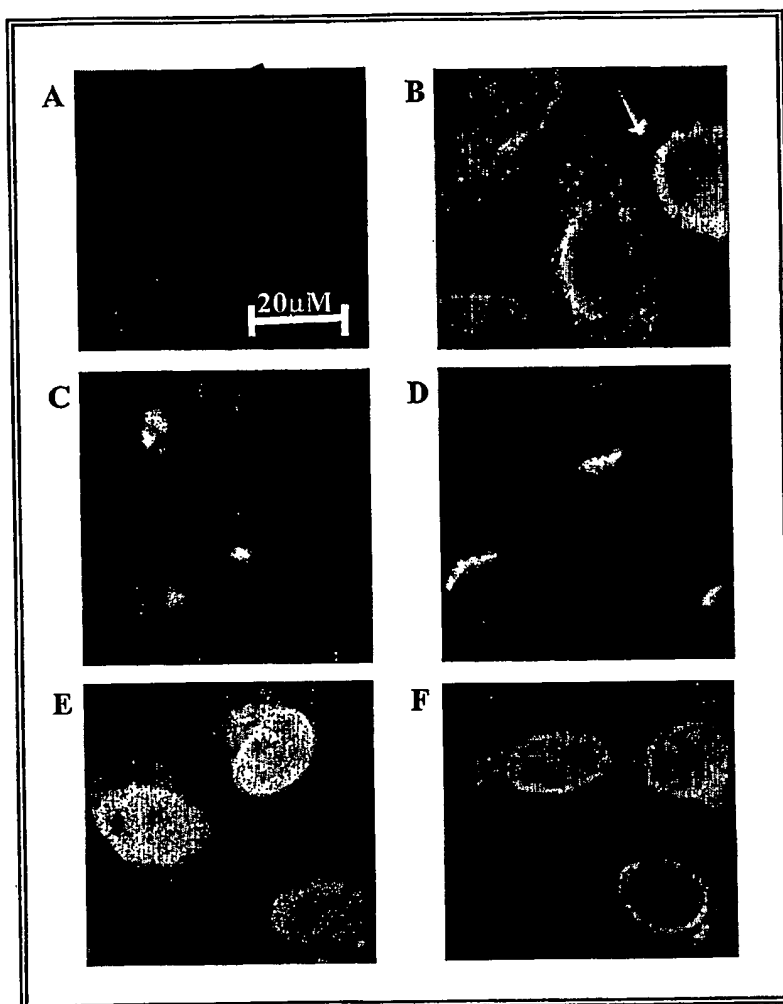


Figure 23.

Figure 1: Human POSH Coding Sequence (SEQ ID NO:1)

ATGGATGAATCAGCCTTGTGGATCTTTTGGAGTGTCCGGTGTGTCTAGAGCGCCTTGATGCTTCTGCGA
AGGTCTTGCCCTTGCCAGCATACGTTTGGCAAGCGATGTTTGTCTGGGGATCGTAGGTTCTCGAAATGAACT
CAGATGTCCCCAGTGCAGGACTCTTGTGGCTCGGGTGTGAGGAGCTTCCAGTAACATCTTGCTGGTC
AGACTTCTGGATGGCATCAAACAGAGGCCCTTGGAAACCTGGTCTGTGGGGGAAGTGGGACCAACTGCA
CAAATGCATTAAAGTCTCAGAGCAGCACTGTGGCTAATTGTAGCTCAAAAGATCTGCAGAGCTCCAGGG
CGGACAGCAGCCTCGGGTGAATCCTGGAGCCCCCAGTGAGGGGTATACCTCAGTTACCATGTGCCAAA
GCGTTATACAACCTATGAAGGAAAAGAGCCTGGAGACCTTAAATTGAGCAAGGCGACATCATCATTTTGC
GAAGACAAGTGGATGAAAATTGGTACCATGGGGAAGTCAATGGAATCCATGGCTTTTCCCCACCAACTT
TGTGCAGATTATTAAACCGTTACCTCAGCCCCCAGTCAAGCAAGCACTTTATGACTTTGAAGTGAAA
GACAAGGAAGCAGACAAAGATTGCCTTCCATTGCAAGGATGATGTTCTGACTGTGATCCGAAGAGTGG
ATGAAAACCTGGGCTGAAGGAATGCTGGCAGACAAAATAGGAATATTTCCAATTTTATATGTTGAGTTTAA
CTCGGCTGCTAAGCAGCTGATAGAATGGGATAAGCCTCCTGTGCCAGGAGTTGATGCTGGAGAATGTTCC
TCGGCAGCAGCCCAGAGCAGCACTGCCCCAAAGCACTCCGACACCAAGAAGAACACCAAAAAGCGGCACT
CCTTCACTTCCCTCACTATGGCCAAAGTCTTCCAGGCATCCAGAACCGCCACTCCATGGAGATCAG
CCCCCTGTCTCTCAGCTCCAGCAACCCCACTGTGCTGCACGGATCAGCGAGCTGTCTGGGCTCTCC
TGCAGTGCCCTTCTCAGGTTTATATAAGTACCACCGGGTTAATTGTGACCCCGCCCCAAGCAGCCCAG
TGACAACTGGCCCCCTCGTTTACTTTCCCATCAGATGTTCCCTACCAAGCTGCCCTTGGAACCTTTGAATCC
TCCTCTTCCACCACCCCTCTCCTGGCTGCCACTGTCTTGCCTCCACACCACAGGCGCCACCGCCGCC
GCTGCTGCTGCTGGAATGGGACCGAGGCCCATGGCAGGATCCACTGACCAGATTGCACATTTACGGCCGC
AGACTCGCCCCAGTGTGTATGTTGCTATATATCCATACACTCCTCGGAAAGAGGATGAACTAGAGCTGAG
AAAAGGGGAGATGTTTTTAGTGTGTTGAGCGCTGCCAGGATGGCTGGTTCAAAGGGACATCCATGCATACC
AGCAAGATAGGGGTTTTCCCTGGCAATTATGTGGCACCAGTCACAAGGGCGGTGACAAATGCTTCCCAAG
CTAAAGTCCCTATGTCTACAGCTGGCCAGACAAGTCGGGGAGTGACCATGGTCAGTCTTCCACGGCAGG
AGGGCCTGCCCAGAAAGCTCCAGGAAATGGCGTGGCTGGGAGTCCCAGTGTGTCCCCGAGCTGTGGTA
TCAGCAGCTCACATCCAGACAAGTCTCAGGCTAAGGTCTTGTGTCATGACGGGGCAAATGACAGTCA
ACCAGGCCCGCAATGCTGTGAGGACAGTTGCAGCGCACACCAGGAACGCCCCACGGCAGCAGTGACACC
CATCCAGGTACAGAATGCCGCCGGCCTCAGCCCTGCATCTGTGGGCCTGTCCCATCACTCGCTGGCCTCC
CCACAACCTGCGCCTCTGATGCCAGGCTCAGCCACGCACACTGCTGCCATCAGTATCAGTCGAGCCAGTG
CCCCCTGCGCCTGTGACGAGCTGCTCCACTGACTTCCCCAAGCATCACCAGTGCTTCTCTGGAGGCTGA
GCCCAGTGGCCGGATAGTGACCGTTCTCCCTGGACTCCCCACATCTCCTGACAGTGCTTTCATCAGCTTGT
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CATATGA

Figure 2: Human POSH Amino Acid Sequence (SEQ ID NO:2)

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GSCPVDGDGPVTTAVAGAALAQDAFHRKASSLDSAVPIAPPPRQACSSLGPVLNESRPVVCERHRVVVSY
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Figure 3: Human POSH cDNA Sequence (SEQ ID NO:3)

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Figure 4: 5' cDNA fragment of human POSH (public gi:10432611; SEQ ID NO:4)

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Figure 5: N terminus protein fragment of hPOSH (public gi:10432612; SEQ ID NO:5)

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GNSSATKPKDKS

Figure 6: 3' mRNA fragment of hPOSH (public gi:7959248; SEQ ID NO:6)

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
Figure 7: C terminus protein fragment of hPOSH (public gi:7959249; SEQ ID NO:7)


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PGSFVENI

Figure 8: Human POSH full mRNA, Annotated Sequence


----- gi|10432611|dbj|AK021429.1|AK021429 Homo sapiens cDNA FLJ11367 fis, clone HEMBA1000303, highly similar to Mus musculus Plenty of SH3s (POSH) mRNA



----- gi|7959248|dbj|AB040927.1|AB040927 Homo sapiens mRNA for KIAA1494 protein, partial cds


 - Both hPOSH and KIAA1495

 - Ring Domain













 - SH3 Domian

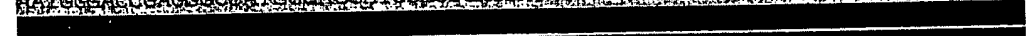














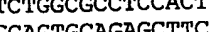
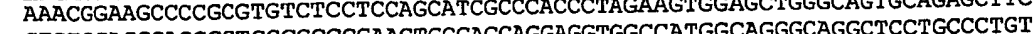
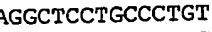




 - start codon and stop codon of predicted ORF

CTGAGAGACACTGCGAGCGGCGAGCGGGTGGGGCCGCATCTGCATCAGCCGCCGAGCCGCTGCGGGGC
CGCGAACAAAGAGGAGGAGCGGAGGCGGAGAGCAAAGTCTGAAATGGATGTTACATGAGTCATTTAAG
GGATGCACACAATATGAACATTTCTGAAGATTTTCTCAGTAAAGTAGATAAAG  GATGAATCAGC
CTTGTGGATCTTTGGAG 

GCAGGACTCTTGTGGCTCGGGTGTGAGGAGCTTCCAGTAACATCTTGCTGGTCAGACTTCTGGATGG
CATCAACAGAGGCCCTTGGAAACCTGGTCTGGTGGGGGAAGTGGGACCAACTGCACAAATGCATTAAAG
TCTCAGAGCAGCACTGTGGCTAATTGTAGCTCAAAAGATCTGCAGAGCTCCAGGGCGGACAGCAGCCTC
GGGTGCAATCCTGGAGCCCCCAGTGAGGGGTATACCTCAGTTA 

 A
AACCGTTACCTCAGCCCCCA 

GGAGACT
GACACTGAAGAAGCTTAAATCACTTCACACAACAAAGTAGCACAAAGCAGTTTAAACAGAAAGAGCACAT
TTGTGGACTTCCAGATGGTCAGGAGATGAGCAAAGGATTGGTATGTGACTCTGATGCCCCAGCACAGTTA
CCCCAGCGAGCAGAGTGAAGAAGATGTTTGTGTGGGTTTTGTTAGTCTGGATTCCGGATGTATAAGGTGTG
CCTTGTACTGTCTGATTTACTACACAGAGAACTTTTTTTTTTTTAAAGATATATGACTAAAATGGACA
ATTGTTTTACAAGGCTTAACATAATTTATTTGCTTTTTTAACTTGAACTTTTCGTATAATAGATACGTTCT
TTGGATTATGATTTTAAGAAATTATTAATTTATGAAATGATAGGTAAGGAGAAGCTGGATTATCTCCTGT
TGAGAGCAAGAGATTTCGTTTTGACATAGAGTGAATGCATTTTCCCCTCTCCTCCTCCTGCTACCATTAT
ATTTTGGGGTTATGTTTTGCTTCTTTAAGATAGAAATCCAGTTCTCTAATTTGGTTTTCTCTTTGGGA
AACCAAACATACAAATGAATCAGTATCAATTAGGGCCTGGGGTAGAGAGACAGAACTTGAGAGAAGAGA
AGTTAGTGATTCCCTCTCTTTCTAGTTTGGTAGGAATCACCCCTGAAGACCTAGTCCCTCAATTTAATTGTG
TGGGTTTTTAATTTTCTAGAAATGAAGTGACTGAAACAATGAGAAAGAATACAGCACAAACCCTTGAACAA
AATGATTTAGAAATATATTTAGTTTTATAGCAGAAGCAGCTCAATTGTTTGGTTGGAAAGTAGGGGAAA
TTGAAGTTGTAGTCACTGTCTGAGAATGGCTATGAAGCGTCATTTACATTTTACCCCACTGACCTGCA
TGCCCAGGACACAAGTAAACATTTGTGAGATAGTGGTGGTAAGTGATGCACCTGTTAAGTCAAAGGC
TATAAGAAACACTGTGAAAAGTTCATATTCATCCATTGTGATTTCTTTCCCACGCTCTTGCAATGATTACT
GGATTCCCACAGTAATATAGACTGTGCATGGTGTGTATATTTCAATTGCGATTTCCTGTTAAGATGAGTTT
GTACTCAGAATTGACCAATTCAGGAGGTGTAAAAATAAACAGTGTCTCTTCTTACCCCAAAGCCACTA
CTGACCAAGGTCTCTTCAGTGCACTCGCTCCCTCTCTGGCTAAGGCATGCATTAGCCACTACACAAGTCA
TTAGTGAAAGTGGTCTTTTTATGTCTCCAGCAGACAGACATCAAGGATGAGTTAACCAGGAGACTACTC
CTGTGACTGTGAGCTCTGGAAGGCTTGGTGGGAGTGAATTTGCCACACCTTACAATTGTGGCAGGATC
CAGAAGAGCCTGTCTTTTTATATCCATTCTTGATGTCATTGGCCTCTCCACCGATTTCAATACGGTGC
CAGCAGTCATGGATCTGGGTAGTCCGGAAAAACAAAGGAGGGAAGACAGCCTGGTAATGAATAAGATCC
TTACCACAGTTTTCTCATGGGAAATACATAATAAACCTTTTCATCTTTTTTTTTTCTTTAAGAATTAA
AACTGGGAAATAGAAACATGAACGTGAAAAGTCTTGAATGACAAGAGGTTTCATGGTCTTAAAAAGATAC
TTTATATGGTTGAAGATGAAATCATTCCTAAATTAACCTTTTTTTTTAAAAAAAACAATGTATATTATGT
TCCTGTGTGTTGAATTTAAAAAAAATACTTTACTTGGATATTCATGTAATATATAAAGGTTTGGTG
AAATGAACCTTTAGTTAGGAAAAAGCTGGCATCAGCTTTCATCTGTGTAAGTTGACACCAATGTGTCATAA
TATTCTTTATTTTGGGAAATTAGTGTATTTTAAAAAATTTTAAAAAGAAAAAGACTACTACAGGTTAA
GATAATTTTTTTTACCTGTCTTTTCTCCATATTTAAGCTATGTGATTGAAGTACCTCTGTTTCATAGTTTC
CTGGTATAAAGTTGGTTAAAATTTTCATCTGTTAATAGATCATTAGGTAATATAATGTATGGGTTTTCTAT
TGGTTTTTTGCAGACAGTAGAGGGAGATTTTGTAAACAAGGGCTTGTACACAGTGATATGGTAATGATAA
AATTGCAATTTATCACTCCTTTTCATGTTAATAATTTGAGGACTGGATAAAAGGTTTCAAGATTTAAATT
TGATGTTCAAACCTTTGT

Figure 9: Domain Analysis of Human POSH

Domain Name	begin	end	E-value
<u>RING</u>	12	52	1.06e-08
<u>SH3</u>	137	192	2.76e-19
<u>SH3</u>	199	258	4.84e-15
<u>low complexity</u>	366	384	-
<u>low complexity</u>	390	434	-
<u>SH3</u>	448	505	2.40e-19
<u>low complexity</u>	547	563	-
<u>low complexity</u>	652	668	-
<u>low complexity</u>	705	729	-
<u>SH3</u>	832	888	1.47e-14

Figure 10: Diagram of Human POSH Nucleic Acids

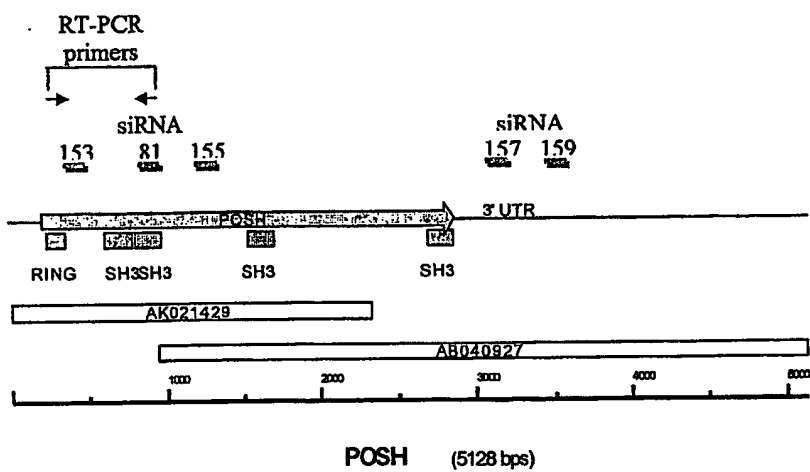


Figure 11: Reduction in Full Length POSH mRNA by siRNA Duplexes

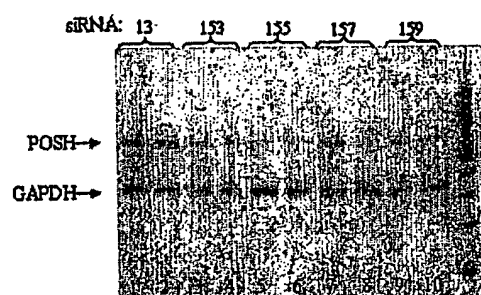
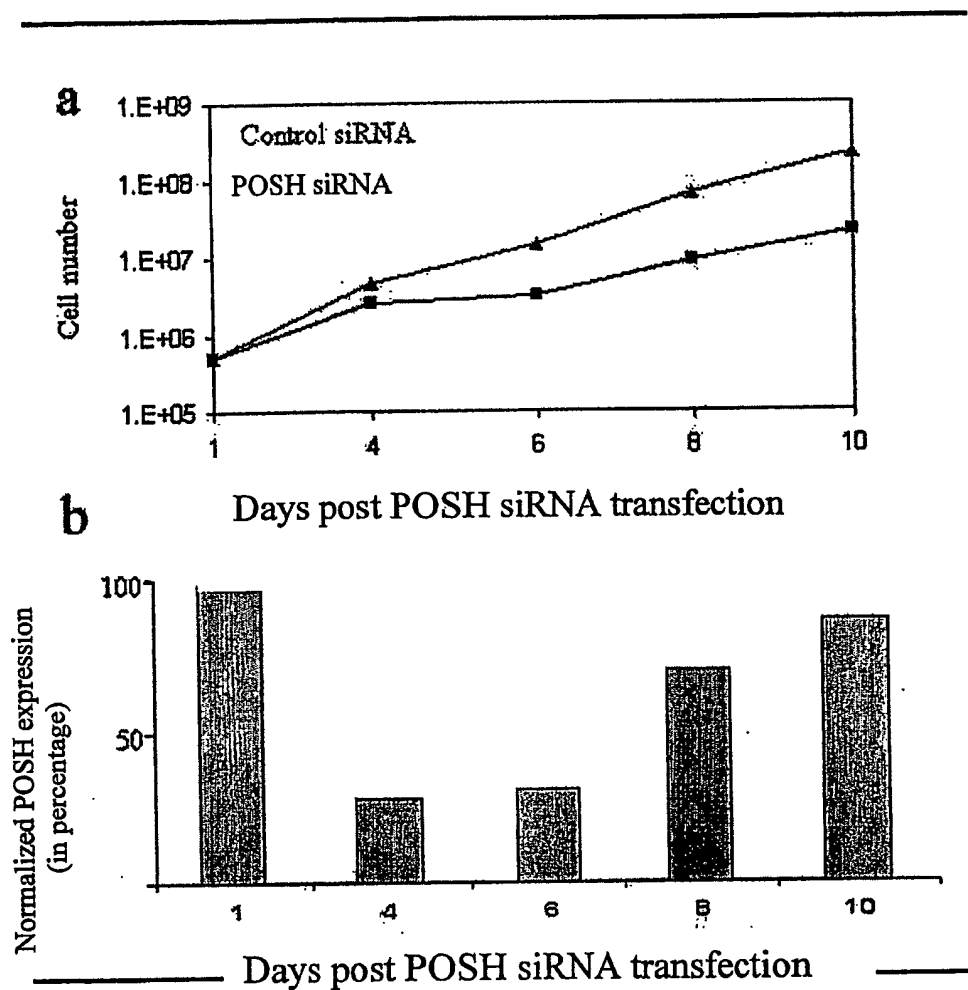


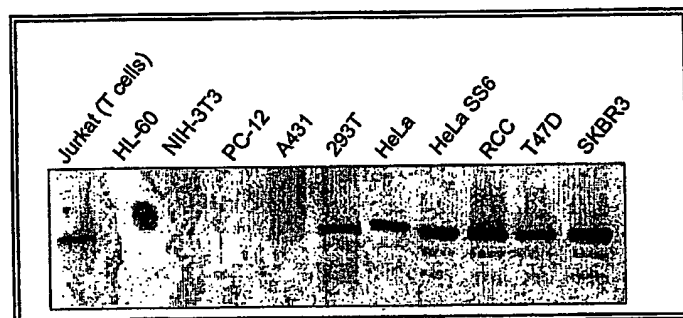
Figure 12



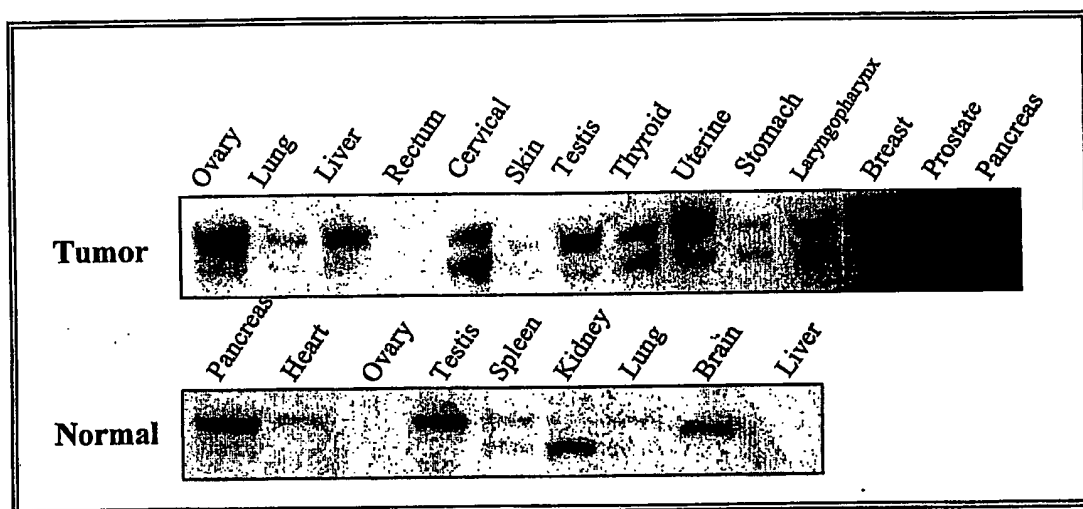
a. Growth curve of HeLa SS cells as a function of time.

b. Levels of POSH expression as a function of time after POSH siRNA transfection.

Figure 13



A. Expression profile of POSH protein in different cell lines.



B. Tissue-specific expression of POSH protein.

Figure 14: Mouse POSH mRNA sequence (public gi:10946921; SEQ ID NO: 8)

GGGACGCGGGCTCGGCGGGGCTGCATCTACCAGCGCTGCGGGGCGCGAAACAAAGGCAGCAGCGGAGGC
GCGAGAGCAAAGTCTGAAATGGATGTTACATGAATCACTTTAAGGGCTGCGCACAACATATGAACGTTCTG
AAGCCGTTTTCTCACTAAAGTCACTCAAGATGGATGAGTCTGCCTTGTGGACCTTCTGGAGTGCCTGTG
GTGTCTAGAACGCCTGGATGCTTCCGCAAAAGGTCTTACCCTGCCAGCATACCTTTTGGCAAACGCTGTTTG
CTGGGGATTGTGGGTTCCCGGAATGAACCTCAGATGTCCCGAATGCCGGACTCTTGTGGCTCTGGGGTGG
ACGAGCTCCCCAGTAACATCTACTGGTCACTTCTGGATGGCATCAAGCAGAGGCCTTGGAAACCCGG
CCCTGGTGGGGCGCGGAGCACTCTGCACAAAACATTAAGGGCGCAGGGCAGCACTGTGGTTAATTGT
GGCTCGAAAGATCTGCAGAGCTCCAGTGTGGACAGAGCCTCGGGTGCAGCCTGGAGCCCCCAGTGA
GGGGAATACCTCAGTTACCGTGTGCCAAAGCATTATATAACTACGAAGGAAAGAGCCCCGAGACCTTAA
GTTCAGCAAAGGCGACACCATCATTCTGCGCCGACAGGTGGATGAGAATTGGTACCACGGGGAAGTCAGC
GGGTCCACGCTTTTTCCCCACTAACTTCGTGCAGATCATCAAACCTTTACCTCAGCCCCCGCTCAGT
GCAAAGCACTTTACGACTTTGAAGTGAAGACGAAGGAGTGAAGAAGATTGCCTTCCCTCGCAAAGGA
GCACGTACTGACCGTGATCCGACAGTGGATGAAACTGGGCTGAAGGAATGCTGGCAGATAAAATAGGA
ATATTTCCAATTTTCATACGTGGAGTTTAACTCAGCTGCCAAGCAGCTGATAGAGTGGGATAAGCCTCCCG
TGCCAGGAGTGGACACGGCAGAATGCCCCCTCAGCGACGGCGCAGAGCACCTCTGCCTCAAAGCACCCCGA
CACCAAGAAGAACACCAGGAAGCGACACTCCTTCACCTCCCTCACCATGGCCAAACAAGTCTTCCAGGGG
TCCCAGAACCGCCACTCCATGGAGATCAGCCCTCCTGTGCTCATCAGTTCAGCAACCCACAGCCGAG
CCCCCATCAGCGAACTGTCCGGGCTCTCTGTCAGCGCCCCGCTCAGGTCATATAAGCACCACTGGGT
AATTGTGACCCCAACCCCTAGCAGCCCGGTGACAACTGGCCCTCGCTTCACTTCCCTTCAGATGTCCCC
TACCAAGCTGCCCTTGAAGATGAACTCTCACTTCCCCCAACCCCTCTCTTGGCGGCCACCGTACTCG
CCTCAGCAACCCCGTCAGGCGCTACTGCTGCTGTTGCTGCTGCTGCTGCCGCGCCGCGCGCTGCTGGAATGGG
ACCCAGGCCTGTGATGGGGTCTCTGAACAGATTGCACATTTACGGCCTCAGACTCGTCCCAGTGTATAT
GTTGCTATATATCCGTACACTCCCCGGAAGGAAGACGAACCTGGAGCTGAGGAAAGGGGAGATGTTTTTG
TGTTTGAGCGTTGCCAGGACGGCTGGTACAAAGGGAACATCGATGCATACCAGCAAGATAGGCGTTTTCC
TGGCAACTATGTGGGCGCCGTCAAGAGGGCGGTGACGAATGCTTCCCAAGCTTAAAGTCTCTATGTCTACT
GCGGCTCAGGCAAGTGCGGGGTGACCATGCTCAGCCCTTCACTGCAGGAGGACCTACACAGAAGCCCC
AAGGAAACGGCGTGGCCGGAAATCCCAGCGTCTCCCCACGGCTGTGGTGTGACGAGCTCATATCCAGAC
AAGTCTCAGGCTAAGGTCTGCTGCACATGTCTGGGCAGATGACAGTCAATCAGGCCCGCAATGCTGTG
AGGACAGTTGCAGCATATAGCCAGGAACGCCCCACAGCAGCAGTGAATCCCATCAGGTCCAGAATGCCG
CCTGCCTTGGTCTGTCATCCGTGGGCTGCCCCATCATTTCTTGGCTCCCCAACCTCTGCCTCCAATGGC
GGGTCTCTGCTGCCACGGTGCTGCCGTGACATCAGTGCAGCAATGCCCATGGCCTGCGCTGCAGGG
GCTTCTCTGGCTCCCCAAATATGACCAAGTGCATGTTGGAGACAGAGCCAGTGGTGCACAGTGCACCA
TCTCTCCTGGACTCCCCATATCTCCAGAGAGTGTGTCATCAGCGTGTGGGAACAGTTCAGCTGGGAAACC
AGACAAGGACAGTAAGAAAGAAAAAAGGGCCTACTGAAGCTGCTTTCTGGTGCCTCCACCAAACGCAAG
CCCCGAGTCTCCCTCCAGCATCACCTACCTGGATGTGGAGCTGGGTGCTGGGGAGGCTCCCTTGCAGG
GAGCAGTAGGTCTGAGCTGCCGTAGGGGGCAGCCACGGCAGAGTGGGGTCATGCCCAAGATGGTGA
TGGTCCAGTGGCCGCTGGAACAGCAGCCCTAGCCAGGATGCTTCCACCGCAAGACAAGCTCCCTGGAC
TCCGAGTGGCCATTGCTCCACCACCTCGCCAGGCTGCTCTCCTGGGCCCATGTCATGAATGAGGCC
GGCCTGTGTTTTGTGAAAGGCACAGGTTGGTGGTTTCTACCTCCTCAGAGTGAGGCCGAACCTGAACT
CAAGGAAGGAGATATTGTGTTTGTTCATAAGAAACGAGAGGACGGCTGGTTCAAAGGCACGTTACAGAGG
AATGGGAAGACTGGCCTTTTCCAGGGAGCTTTGTGGAAGACATCTGAGAAGACGGGACACGGAGAAAGC
TTATCATACACCAGTGTGACTAAAGAGCACAAAGCAGTTTCATAGAAAGAGCACATCTGTGGACTTCC
AGATCTTCAAGAACCGAGCAGAAGATGGGCACCTGACTCCAGAGCCCCGGCTGGTTACCCAGGGGACG
AGGGAAGGAGGACAGCACTGTGGTTCGCTCTCTGGGTTCTGATGTGAAGTGTGCTTGTGTAATG
TCTAATGGACTTTACAGATAAAATGCTTTTTTTTTTAAAGATGTATAACTAAAATGACAAATTGTTTAC
AGGCTTAACTAATTTATTTGCTTTTTTAAAACTTGAACCTTCTTGTAAATAGCAAT

Figure 15: Mouse POSH Protein sequence (Public gi: 10946922; SEQ ID NO: 9)

MDESALLDLLECPVCLERLDASAKVLPCHTFCKRCLLGI VGSRNELRCPECRTLVGSGVDELPSNILLV
RLLDGIKQRPWKPGPGGGGGTTCTNTLRAQGSTVVNCGSKDLQSSQCGQPRVQAWSPPVRGIPQLPCAK
ALYNYEGKEPGDLKFSKGDITILRRQVDENWYHGEVSGVHGFFPTNFVQIIKPLPQPPPQCKALYDFEVK
DKBADKDCLPFAKDDVLTVIIRVDENWAEGLADKIGIFPISYVEFN SAAKQLIEWDKPPVPGVDTAECF
SATAQSTSASKHPDTKKNTRKRHSFTSLTMANKSSQGSQNRHSMEISPPVLISSNPTAAARISELSGLS
CSAPSQVHISTTGLIVTPPPSSPVTGPAFTFPSDVPIYQAALGSMNPPLPPPPLAATVLASTPSGATAA
VAAAAAAAAAAGMGRPFVMGSSEQIAHLRPQTRPSVYVAIYPYTPRKEDELELRKGEMFLVFERCQDGWY
KGTSMHTSKIGVFPGNYPVAPVTRAVTNASQAKVSMSTAGQASRGVTMVSPSTAGGPTQKPQGNVAGNPS
VVPTAVVSAAHIQTSFPAKVLHMSGQMTVNQARNAVRTVAHNSQERPTAAVTPIQVQNAACLGPA SVGL
PHHSLASQPLPPMAGPAAHGA AVSISRTNAPMACAAGASLASPNMTSAMLETEPSGRTVTILPGLPTSPE
SAASACGNSSAGKPKDKSKKEKKGLLKL LSGASTKRKPRVSPASP TLDELGAGEAPIQGAVGPELPLG
GSHGRVGCPTDGDGPVAAGTAALAQDAFHRKTSLSAVPIAPPPRQACSSLGPMNEARPVVCERHRV
VVSYPQSEAELELKEGDIVFVHKKREDGWFKGTLQRNGKTGLFPGSFVENI

Figure 16: *Drosophila melanogaster* POSH mRNA sequence (public gi:17737480; SEQ ID NO:10)

CATTTGTATCCGCTTGGCCACGAGCTTTGGCTGCACCTTGGCAAACCTTAATAAATTAAACATTGAATCCTG
CCTATTGCAACGATAATAATCTGATTAGTGCAATTAAGAACGACAAGTAGCGATTATAATAGTAGATT
TTAGCATTTGAGCTAAATTTATTTCCCAACCGCGTCTTGGGATTGCGTATGCGTGAGCCAGTACCTGCAT
GTGTGTGTGTTTGGAAATGTGGCCCTGCACGAAATCAAATAGTGACCATCCTTGAGATTTTGCATACTG
GCAAGATGGACGAGCACACGTTAAACGACCTGTTGGAGTGCTCCGTGTGTCTTGAGCGACTGGACACCAC
ATCGAAGGTGCTGCCATGCCAGCACACCTTCTGCGCAAATGCTTGCAAGACATTGTGGCCAGTCAGCAC
AAGTTGCGATGCCCGGAGTGCCGCACTCTGGTCTCTTGCAAAATTGATGAGCTGCCTCCAAACGTCTTGC
TGATGCGAATCTTAGAAGGCATGAAACAAAATGCAGCAGCTGGCAAAGGAGAAGAAAAGGAGAGAGAC
TGAAACACAGCCGGAAAGGGCCAAACCTCAGCCGCCAGCGGAATCAGTGGCCCCGCTGACAACCAACTA
CTCCAGCTGCAGTCACATCAGCAATCTCATCAGCCGGCTCGTCACAAGCAACGTCGATTTCTACTCCCC
ACGCCTATGCCCTCTTGACTTCGCCTCCGGTGAAGCCACCGATCTAAAGTTCAAGAAAGGGGATCTGAT
ACTGATCAAGCATCGCATCGACAACACTGGTTTGTGGGTCAAGCGAATGGTCAGGAGGGCACATTTCCC
ATCAACTACGTCAAGGTATCGGTTCCGCTGCCCATGCCGAGTGCATTGCCATGTATGACTTTAAGATGG
GGCCCAACGACGAGGAGGGATGCCTCGAATTTAAGAAAAGCACTGTAATACAGGTAATGCGCCGAGTTGA
TCATAATTGGGCAGAAGGACGAATTGGCCAGACCATCGGAATCTTTCCAATAGCATTCTGTTGAGCTGAAT
GCAGCGGCCAAAAGCTGTGGACAGCGGGCTACACACCCATCCATTCTGCCATCCACCGAAGCAACAGG
GGCAGCGGGGCCCTTCCCTCCGGTTCCAGTTATTGATCCACGGTGGTCACGGAATCCAGTTCGGGATCCTC
CAATTCACGCGCGGGCAGCAGCAATTCAAGCTCCACATCCAGCTCGAATAACTGCAGTCCGAATCACCAA
ATCTCACTGCCGAATACCCCCCAACATGTAGTAGCTTCCGATCGGCGTCTGTTCTGTTTCCGTGACAAGG
GAGCAAAGGAGAAACGCCACTCACTAAATGCTTTGCTGGGAGGAGGAGCTCCATTAAGTCTGCTGCAGAG
CAACCGCCATTCCGGCTGAAATTTCTAGCCTGCCCCATGAACTAAGCCGCTTGGAAAGTTTCCAGCTCAACA
GCTCTAAAACCCACGTAGCCCCACAGACATCGCGTGTACTTAAGACCACTGTTTACGACGAGATGCAAC
CGAATTTACCCTGGGGATACTTAGCCCTGTTCCCATACAAACACGCCAAACGGATGAGCTGGAATTAATA
AAAGGGTTGTGTTTACATTGTGACCGAAGCATGTGTGGACGGTTGGTTCAAGGGAAAAAAGCTGTTGGAC
ATCACTGGAGTGTTCCTGGGCAACTACCTGACGCCCCCTGCGCGCCCGCAGCAGCAGTAAATGCATC
AATGGAATATGTTCCCCAAATGCAGACGCCAGATGGCACAAGTACAGCAGCATCCAGTTGCACCAGA
TGTGCGACTCAACAACATGCTGTCCATGCAACCGCTGATTGGCACCTCGTCAGCAGCAGGCTACCGCC
ACGACCACCAAGTTGCTCTGTGTGGTTCGAAACCAAGTGGAGGCGCTGTTTACGACGAGAAATCGGAGCCCAAGC
CTGAACTGCCACAGCTTCGACTACGAGCAGCAGTTCCTCTGAGCAGTGGGACTTATGAGGAGATTAAC
TCACATGAAAAACAGCTCCAAATCTCCGGGAGCGTCCTTGAGCAAGTTCGAAAGAAGCTATTAGCACA
AATGTGGAATTTACAACAAACCCATCAGCTAAATGCATCCAGTACATGTAAGATCCGGCTCGTCCCCCA
GTCAGCTGCAGCAGTCAACCGCTCAATGAAATCCAGCAGCCAAAGACAGCGGCACAACAACAGCAGTT
CCTACCCAAGCAGCTGCCTTCCGCTTCTACGAACAGCGTTTCGTACGGATCGCAACGCGTGAAAGGAAGC
AAGGAACGTCCTCACTTGATTGTCGCGAGACAATCATTAGATGCAGCTACATTTGCGAGTATGTACAACA
ATGCCGCGTCGCGCCGCCACCTACTACTTCCGTGGCCCCAGCTGTCTACGCCGCGGTGAGCAACAGGT
GATTCTTGAGGTGGAGCGCAATCCAGTTGCATGCCAATATGATTATTGCACCCAGCCATCGGAAGTCG
CACAGCCTAGATGCGAGTCATGTGCTGAGTCCAGCAGCAATATGATCACGAGGCGGCCATTAAAGGCCA
GCGCCACCCTAAGTCTCTTACTGCACGAGGGAAAGTCGATTCCGCTGCATTGTGCCGTATCCACCAA
CAGTGACATTGAACTAGAGCTACATTTGGGCGACATATCTACGTCAGCGGAAGCAGAAGAAGCGCTGG
TATAAGGGCACCCATGCCCGTACCCACAAAACCGGGCTGTTCCCCGCTCCTTTGTTGAACCGGATTGTT
AGGAAAGTTATGGTTCAAACTAGAATTTATTAAGCGAAATTCAAATTAAGTTGTTCTAAAAGGATTCAATC
GTCGGTCTATTCCGGCTTCCAAATACGCAATCTCATATTTCTCTTTTCAAAAAGAAACCGTTTTGTACT
CTTCCAATCGAATGGGAGCTCGCCGTTGTAATTTTTATACAATGCTTGATCAAAATAGGCTAGCCATG
TAAGACTTAGGGAACAGTTACTTAAGCCTTAGCGATTAGTTAGCTAGAGAAATAATCTAACCGATCCTTG
TGCCCTCTACAAAGTTATTTGTAATATACGATACTCAGTAATAAAAAAAAAAAAAAAAAAAAAAAAAA

Figure 17: *Drosophila melanogaster* POSH protein sequence (public gi:17737481; SEQ ID NO:11)

MDEHTLNDLLECSVCLERLDTTSKVLPQHTFCRKCLQDIVASQHKLRCECRILVSKIDELPPNVLLM
RILEGMKQNAAAGKGEEKGEETETQPERAKPQPPAESVAPPDNQLQLQSHQQSHQPARHKQRRFLLPHA
YALFDFASGEATDLKFKKGDLLIKHRIDNNWFVGQANGQEGTFPINYVKVSVPLPMPQCIAMYDFKMGF
NDEEGCLEFKKSTVIQVMRRVDHNWAEGRIGQTIGIFPIAFVELNAAAKLLDSGLHHPFCHPPKQQGQ
RALPPVPVIDPTVVTESSSGSSNSTPGSSNSSSTSSSNCSPNHQISLPNTPQHVVASGSASVRFRDKGA
KEKRHSINALLGGGAPLSLLQTNRHSAEILSLPHELSRLEVSSSTALKPTSAPQTSRVLKTTVQQQMOPN
LPWGYLALFPYKPRQTDELELKKGCYIIVTERCVDGWFKGNWLDITGVFPGNYLTPLRARDQQQLMHQW
KYVPQNADAQMAVQQHPVAPDVRLNNMLSMQPPDLPPRQQQATATTTSCSVWSKPVEALFSRKSEPKPE
TATASTSSSSSGAVGLMRRLTHMKTRSKSPGASLQQVPKEAISTNVEFTTNPSAKLHPVHVRSRSGCPSQ
LQHSQPLNETPAAKTAAQQQFLPKQLPSASTNSVYGSQRVKGSKERPHLICARQSLDAATFRSMYNNA
ASPPPTTSVAPAVYAGGQQQVIPGGGAQSQLHANMIIAPSHRKSHSLDASHVLSPPSSNMITEAAIKASA
TTKSPYCTRESRFRCIVPYPPNSDIELELHLGDIYVQRKQKNGWYKGTHARTHTKGLFPASFVEPDC

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Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN, MEDLINE**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TAPON et al. A new Rac target POSH is an SH3-containing scaffold protein involved in the JNK and NF-KB signaling pathways. EMBO J. 1998, Vol. 17, No. 5, pages 1395-1404, especially page 1397-1398.	1-5, 7
A	Database GENESEQ on STN, (Derwent, London, UK) Accession No. AAB93713, 07 February 2001, 100% identity to SEQ ID NO:5.	2, 53

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search

20 May 2004 (20.05.2004)

Date of mailing of the international search report

27 JUL 2004

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/08194

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.: 1-7,25-37 and 52-54 (in-part)
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☒
☐

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group 1, Claims 1-7, drawn to the special technical feature of a method for identifying an antiproliferative agent comprising a POSH polypeptide and a test agent that interacts with the POSH polypeptide wherein the POSH polypeptide is SEQ ID NO:2 or a fragment thereof comprising at least 20 consecutive amino acids.

Group 2, Claims 1-7, drawn to the special technical feature of a method for identifying an antiproliferative agent comprising a POSH polypeptide and a test agent that interacts with the POSH polypeptide wherein the POSH polypeptide is SEQ ID NO:5 or a fragment thereof comprising at least 20 consecutive amino acids.

Group 3, Claims 1-7, drawn to the special technical feature of a method for identifying an antiproliferative agent comprising a POSH polypeptide and a test agent that interacts with the POSH polypeptide wherein the POSH polypeptide is SEQ ID NO:7 or a fragment thereof comprising at least 20 consecutive amino acids.

Group 4, Claims 1-7, drawn to the special technical feature of a method for identifying an antiproliferative agent comprising a POSH polypeptide and a test agent that interacts with the POSH polypeptide wherein the POSH polypeptide is SEQ ID NO:9 or a fragment thereof comprising at least 20 consecutive amino acids.

Group 5, Claims 1-7, drawn to the special technical feature of a method for identifying an antiproliferative agent comprising a POSH polypeptide and a test agent that interacts with the POSH polypeptide wherein the POSH polypeptide is SEQ ID NO:11 or a fragment thereof comprising at least 20 consecutive amino acids.

Group 6, Claims 8-10, drawn to the special technical feature of a method for identifying an antiproliferative agent comprising providing a polypeptide involved in a POSH-mediated cellular proliferation and a test agent wherein the polypeptide involved in a POSH-mediated cellular proliferation is a polypeptide comprising a RING domain and a SH3 domain.

Group 7, Claims 8-10, drawn to the special technical feature of a method for identifying an antiproliferative agent comprising providing a polypeptide involved in a POSH-mediated cellular proliferation and a test agent wherein the polypeptide involved in a POSH-mediated cellular proliferation is Brca1.

Group 8, Claims 8-10, drawn to the special technical feature of a method for identifying an antiproliferative agent comprising providing a polypeptide involved in a POSH-mediated cellular proliferation and a test agent wherein the polypeptide involved in a POSH-mediated cellular proliferation is Rac.

Group 9, Claims 8-10, drawn to the special technical feature of a method for identifying an antiproliferative agent comprising providing a polypeptide involved in a POSH-mediated cellular proliferation and a test agent wherein the polypeptide involved in a POSH-mediated cellular proliferation is Rac1.

Group 10, Claims 8-10, drawn to the special technical feature of a method for identifying an antiproliferative agent comprising providing a polypeptide involved in a POSH-mediated cellular proliferation and a test agent wherein the polypeptide involved in a POSH-mediated cellular proliferation is Vav.

Group 11, Claims 8-10, drawn to the special technical feature of a method for identifying an antiproliferative agent comprising providing a polypeptide involved in a POSH-mediated cellular proliferation and a test agent wherein the polypeptide involved in a POSH-mediated cellular proliferation is Cdc42.

Group 12, Claims 8-10, drawn to the special technical feature of a method for identifying an antiproliferative agent comprising providing a polypeptide involved in a POSH-mediated cellular proliferation and a test agent wherein the polypeptide involved in a POSH-mediated cellular proliferation is PI3K.

INTERNATIONAL SEARCH REPORT

PCT/US03/08194

Group 13, Claims 11-14, drawn to the special technical feature of a method for identifying an antiproliferative agent comprising providing a POSH nucleic acid and a test agent wherein the POSH nucleic acid is SEQ ID NO:1.

Group 14, Claims 11-14, drawn to the special technical feature of a method for identifying an antiproliferative agent comprising providing a POSH nucleic acid and a test agent wherein the POSH nucleic acid is SEQ ID NO:3.

Group 15, Claims 11-14, drawn to the special technical feature of a method for identifying an antiproliferative agent comprising providing a POSH nucleic acid and a test agent wherein the POSH nucleic acid is SEQ ID NO:4.

Group 16, Claims 11-14, drawn to the special technical feature of a method for identifying an antiproliferative agent comprising providing a POSH nucleic acid and a test agent wherein the POSH nucleic acid is SEQ ID NO:6.

Group 17, Claims 11-14, drawn to the special technical feature of a method for identifying an antiproliferative agent comprising providing a POSH nucleic acid and a test agent wherein the POSH nucleic acid is SEQ ID NO:8.

Group 18, Claims 11-14, drawn to the special technical feature of a method for identifying an antiproliferative agent comprising providing a POSH nucleic acid and a test agent wherein the POSH nucleic acid is SEQ ID NO:10.

Group 19, Claims 15-17, 70-71, drawn to the special technical feature of a method for identifying an antiproliferative agent comprising providing a nucleic acid involved in a POSH-mediated cellular proliferation and a test agent wherein the nucleic acid involved in a POSH-mediated cellular proliferation encodes a polypeptide comprising a RING domain and a SH3 domain.

Group 20, Claims 15-17, 70-71, drawn to the special technical feature of a method for identifying an antiproliferative agent comprising providing a nucleic acid involved in a POSH-mediated cellular proliferation and a test agent wherein the nucleic acid involved in a POSH-mediated cellular proliferation encodes Brca1.

Group 21, Claims 15-17, 70-71, drawn to the special technical feature of a method for identifying an antiproliferative agent comprising providing a nucleic acid involved in a POSH-mediated cellular proliferation and a test agent wherein the nucleic acid involved in a POSH-mediated cellular proliferation encodes Rac.

Group 22, Claims 15-17, 70-71, drawn to the special technical feature of a method for identifying an antiproliferative agent comprising providing a nucleic acid involved in a POSH-mediated cellular proliferation and a test agent wherein the nucleic acid involved in a POSH-mediated cellular proliferation encodes Rac1.

Group 23, Claims 15-17, 70-71, drawn to the special technical feature of a method for identifying an antiproliferative agent comprising providing a nucleic acid involved in a POSH-mediated cellular proliferation and a test agent wherein the nucleic acid involved in a POSH-mediated cellular proliferation encodes Vav.

Group 24, Claims 15-17, 70-71, drawn to the special technical feature of a method for identifying an antiproliferative agent comprising providing a nucleic acid involved in a POSH-mediated cellular proliferation and a test agent wherein the nucleic acid involved in a POSH-mediated cellular proliferation encodes Cdc42.

Group 25, Claims 15-17, 70-71, drawn to the special technical feature of a method for identifying an antiproliferative agent comprising providing a nucleic acid involved in a POSH-mediated cellular proliferation and a test agent wherein the nucleic acid involved in a POSH-mediated cellular proliferation encodes PI3K.

Group 26, Claims 18-21, 24, drawn to the special technical feature of a method for identifying an antiapoptotic agent comprising providing a POSH polypeptide and a test agent, wherein the POSH polypeptide is at least 95% identical to SEQ ID NO:2.

Group 27, Claims 22-24, drawn to the special technical feature of a method for identifying an antiapoptotic agent comprising identifying a test agent that binds to a POSH nucleic acid wherein the test agent is a ribonucleic acid.

Group 28, Claims 25-37, drawn to the special technical feature of a method for inhibiting cellular proliferation in a subject in need thereof, comprising administering an effective amount of an agent that inhibits a POSH polypeptide wherein the agent is selected from the group consisting of antisense, an RNAi construct, a DNA enzyme, and a ribozyme, wherein the RNAi construct is SEQ ID NO:15.

Group 29, Claims 25-37, drawn to the special technical feature of a method for inhibiting cellular proliferation in a subject in need thereof, comprising administering an effective amount of an agent that inhibits a POSH polypeptide wherein the agent is selected from the group consisting of antisense, an RNAi construct, a DNA enzyme, and a ribozyme, wherein the RNAi construct is SEQ ID NO:16.

INTERNATIONAL SEARCH REPORT

PCT/US03/08194

Group 30, Claims 25-37, drawn to the special technical feature of a method for inhibiting cellular proliferation in a subject in need thereof, comprising administering an effective amount of an agent that inhibits a POSH polypeptide wherein the agent is selected from the group consisting of antisense, an RNAi construct, a DNA enzyme, and a ribozyme, wherein the RNAi construct is SEQ ID NO:18.

Group 31, Claims 25-37, drawn to the special technical feature of a method for inhibiting cellular proliferation in a subject in need thereof, comprising administering an effective amount of an agent that inhibits a POSH polypeptide wherein the agent is selected from the group consisting of antisense, an RNAi construct, a DNA enzyme, and a ribozyme, wherein the RNAi construct is SEQ ID NO:19.

Group 32, Claims 25-37, drawn to the special technical feature of a method for inhibiting cellular proliferation in a subject in need thereof, comprising administering an effective amount of an agent that inhibits a POSH polypeptide wherein the agent is selected from the group consisting of antisense, an RNAi construct, a DNA enzyme, and a ribozyme, wherein the RNAi construct is SEQ ID NO:21.

Group 33, Claims 25-37, drawn to the special technical feature of a method for inhibiting cellular proliferation in a subject in need thereof, comprising administering an effective amount of an agent that inhibits a POSH polypeptide wherein the agent is selected from the group consisting of antisense, an RNAi construct, a DNA enzyme, and a ribozyme, wherein the RNAi construct is SEQ ID NO:22.

Group 34, Claims 25-37, drawn to the special technical feature of a method for inhibiting cellular proliferation in a subject in need thereof, comprising administering an effective amount of an agent that inhibits a POSH polypeptide wherein the agent is selected from the group consisting of antisense, an RNAi construct, a DNA enzyme, and a ribozyme, wherein the RNAi construct is SEQ ID NO:24.

Group 35, Claims 25-37, drawn to the special technical feature of a method for inhibiting cellular proliferation in a subject in need thereof, comprising administering an effective amount of an agent that inhibits a POSH polypeptide wherein the agent is selected from the group consisting of antisense, an RNAi construct, a DNA enzyme, and a ribozyme, wherein the RNAi construct is SEQ ID NO:25.

Group 36, Claims 38-48, drawn to the special technical feature of a non-human transgenic animal that comprises a plurality of cells containing one or more recombinant constructs of a POSH gene, wherein expression of said POSH gene mitigates a POSH loss of function phenotype.

Group 37, Claim 49, drawn to the special technical feature of a method of screening for a substance to be used for treating a neoplastic condition comprising administering a test compound to the non-human animal of claim 38, and assaying efficacy of said test compound in potentiating the POSH loss of function phenotype.

Group 38, Claim 50, drawn to the special technical feature of evaluating the antiproliferation potential of an agent comprising contacting a transgenic non human animal of step (i) with a test agent, and (ii) comparing the cellular proliferation status.

Group 39, Claim 51, drawn to the special technical feature of a method of evaluating an anti-proliferative activity of a test compound comprising contacting the non-human transgenic animal or germline and somatic cells expressing a human POSH transgene with a test agent and determining the cellular proliferation in a specimen.

Group 40, Claims 52-53, drawn to the special technical feature of a method of inhibiting cellular proliferation comprising inhibiting a ubiquitin-related activity of a POSH polypeptide wherein the POSH polypeptide is SEQ ID NO:2 or a fragment thereof comprising at least 20 consecutive amino acids.

Group 41, Claims 52-53, drawn to the special technical feature of a method of inhibiting cellular proliferation comprising inhibiting a ubiquitin-related activity of a POSH polypeptide wherein the POSH polypeptide is SEQ ID NO:5 or a fragment thereof comprising at least 20 consecutive amino acids.

Group 42, Claims 52-53, drawn to the special technical feature of a method of inhibiting cellular proliferation comprising inhibiting a ubiquitin-related activity of a POSH polypeptide wherein the POSH polypeptide is SEQ ID NO:7 or a fragment thereof comprising at least 20 consecutive amino acids.

Group 43, Claims 52-53, drawn to the special technical feature of a method of inhibiting cellular proliferation comprising inhibiting a ubiquitin-related activity of a POSH polypeptide wherein the POSH polypeptide is SEQ ID NO:9 or a fragment thereof comprising at least 20 consecutive amino acids.

INTERNATIONAL SEARCH REPORT

PCT/US03/08194

Group 44, Claims 52-53, drawn to the special technical feature of a method of inhibiting cellular proliferation comprising inhibiting a ubiquitin-related activity of a POSH polypeptide wherein the POSH polypeptide is SEQ ID NO:11 or a fragment thereof comprising at least 20 consecutive amino acids.

Group 45, Claims 52, 54, drawn to the special technical feature of a method of inhibiting cellular proliferation comprising inhibiting a ubiquitin-related activity of a POSH polypeptide further comprising inhibiting the RING domain of the POSH polypeptide.

Group 46, Claims 52, 55-56, drawn to the special technical feature of a method of inhibiting cellular proliferation comprising inhibiting a ubiquitin-related activity of a POSH polypeptide comprising administering an agent including an antibody specifically immunoreactive with SEQ ID NO:30.

Group 47, Claims 52, 57-58, 80-84, drawn to the special technical feature of a method of inhibiting cellular proliferation comprising inhibiting a ubiquitin-related activity of a POSH polypeptide comprising administering an agent wherein the agent is selected from the group consisting of antisense, an RNAi construct, a DNA enzyme, and a ribozyme, wherein the RNAi construct is SEQ ID NO:15.

Group 48, Claims 52, 57-58, 80-84, drawn to the special technical feature of a method of inhibiting cellular proliferation comprising inhibiting a ubiquitin-related activity of a POSH polypeptide comprising administering an agent wherein the agent is selected from the group consisting of antisense, an RNAi construct, a DNA enzyme, and a ribozyme, wherein the RNAi construct is SEQ ID NO:16.

Group 49, Claims 52, 57-58, 80-84, drawn to the special technical feature of a method of inhibiting cellular proliferation comprising inhibiting a ubiquitin-related activity of a POSH polypeptide comprising administering an agent wherein the agent is selected from the group consisting of antisense, an RNAi construct, a DNA enzyme, and a ribozyme, wherein the RNAi construct is SEQ ID NO:18.

Group 50, Claims 52, 57-58, 80-84, drawn to the special technical feature of a method of inhibiting cellular proliferation comprising inhibiting a ubiquitin-related activity of a POSH polypeptide comprising administering an agent wherein the agent is selected from the group consisting of antisense, an RNAi construct, a DNA enzyme, and a ribozyme, wherein the RNAi construct is SEQ ID NO:19.

Group 51, Claims 52, 57-58, 80-84, drawn to the special technical feature of a method of inhibiting cellular proliferation comprising inhibiting a ubiquitin-related activity of a POSH polypeptide comprising administering an agent wherein the agent is selected from the group consisting of antisense, an RNAi construct, a DNA enzyme, and a ribozyme, wherein the RNAi construct is SEQ ID NO:21.

Group 52, Claims 52, 57-58, 80-84, drawn to the special technical feature of a method of inhibiting cellular proliferation comprising inhibiting a ubiquitin-related activity of a POSH polypeptide comprising administering an agent wherein the agent is selected from the group consisting of antisense, an RNAi construct, a DNA enzyme, and a ribozyme, wherein the RNAi construct is SEQ ID NO:22.

Group 53, Claims 52, 57-58, 80-84, drawn to the special technical feature of a method of inhibiting cellular proliferation comprising inhibiting a ubiquitin-related activity of a POSH polypeptide comprising administering an agent wherein the agent is selected from the group consisting of antisense, an RNAi construct, a DNA enzyme, and a ribozyme, wherein the RNAi construct is SEQ ID NO:24.

Group 54, Claims 52, 57-58, 80-84, drawn to the special technical feature of a method of inhibiting cellular proliferation comprising inhibiting a ubiquitin-related activity of a POSH polypeptide comprising administering an agent wherein the agent is selected from the group consisting of antisense, an RNAi construct, a DNA enzyme, and a ribozyme, wherein the RNAi construct is SEQ ID NO:25.

Group 55, Claims 59-61, 65-66, drawn to the special technical feature of a therapeutic composition comprising an inhibitor of any one of a POSH polypeptide or a polypeptide set forth in SEQ ID NO:26.

Group 56, Claims 59-62, 65-66, drawn to the special technical feature of a therapeutic composition comprising an inhibitor of any one of a POSH polypeptide or a polypeptide set forth in SEQ ID NO:30.

Group 57, Claims 59, 63-66, drawn to the special technical feature of a therapeutic composition comprising an inhibitor selected from the group consisting of antisense, an RNAi construct, a DNA enzyme, and a ribozyme, wherein the RNAi construct is SEQ ID NO:15.

Group 58, Claims 59, 63-66, drawn to the special technical feature of a therapeutic composition comprising an inhibitor selected from the group consisting of antisense, an RNAi construct, a DNA enzyme, and a ribozyme, wherein the RNAi construct is SEQ ID NO:16.

Group 59, Claims 59, 63-66, drawn to the special technical feature of a therapeutic composition comprising an inhibitor selected from the group consisting of antisense, an RNAi construct, a DNA enzyme, and a ribozyme, wherein the RNAi construct is SEQ ID NO:18.

INTERNATIONAL SEARCH REPORT

PCT/US03/08194

Group 60, Claims 59, 63-66, drawn to the special technical feature of a therapeutic composition comprising an inhibitor selected from the group consisting of antisense, an RNAi construct, a DNA enzyme, and a ribozyme, wherein the RNAi construct is SEQ ID NO:19.

Group 61, Claims 59, 63-66, drawn to the special technical feature of a therapeutic composition comprising an inhibitor selected from the group consisting of antisense, an RNAi construct, a DNA enzyme, and a ribozyme, wherein the RNAi construct is SEQ ID NO:21.

Group 62, Claims 59, 63-66, drawn to the special technical feature of a therapeutic composition comprising an inhibitor selected from the group consisting of antisense, an RNAi construct, a DNA enzyme, and a ribozyme, wherein the RNAi construct is SEQ ID NO:22.

Group 63, Claims 59, 63-66, drawn to the special technical feature of a therapeutic composition comprising an inhibitor selected from the group consisting of antisense, an RNAi construct, a DNA enzyme, and a ribozyme, wherein the RNAi construct is SEQ ID NO:24.

Group 64, Claims 59, 63-66, drawn to the special technical feature of a therapeutic composition comprising an inhibitor selected from the group consisting of antisense, an RNAi construct, a DNA enzyme, and a ribozyme, wherein the RNAi construct is SEQ ID NO:25.

Group 65, Claims 67-69, drawn to the special technical feature of a method of inhibiting disease-associated cellular proliferation comprising administering an agent that inhibits a polypeptide involved in POSH-mediated cellular proliferation wherein the polypeptide is a nucleic acid that encodes a polypeptide comprising a RING domain and a SH3 domain.

Group 66, Claims 67-69, drawn to the special technical feature of a method of inhibiting disease-associated cellular proliferation comprising administering an agent that inhibits a polypeptide involved in POSH-mediated cellular proliferation wherein the polypeptide is a nucleic acid that encodes Brca1.

Group 67, Claims 67-69, drawn to the special technical feature of a method of inhibiting disease-associated cellular proliferation comprising administering an agent that inhibits a polypeptide involved in POSH-mediated cellular proliferation wherein the polypeptide is a nucleic acid that encodes Rac1.

Group 68, Claims 67-69, drawn to the special technical feature of a method of inhibiting disease-associated cellular proliferation comprising administering an agent that inhibits a polypeptide involved in POSH-mediated cellular proliferation wherein the polypeptide is a nucleic acid that encodes Rac1.

Group 69, Claims 67-69, drawn to the special technical feature of a method of inhibiting disease-associated cellular proliferation comprising administering an agent that inhibits a polypeptide involved in POSH-mediated cellular proliferation wherein the polypeptide is a nucleic acid that encodes Vav.

Group 70, Claims 67-69, drawn to the special technical feature of a method of inhibiting disease-associated cellular proliferation comprising administering an agent that inhibits a polypeptide involved in POSH-mediated cellular proliferation wherein the polypeptide is a nucleic acid that encodes Cdc42.

Group 71, Claims 67-69, drawn to the special technical feature of a method of inhibiting disease-associated cellular proliferation comprising administering an agent that inhibits a polypeptide involved in POSH-mediated cellular proliferation wherein the polypeptide is a nucleic acid that encodes PI3K.

Group 72, Claim 72, drawn to the special technical feature of a method for evaluating the anti-proliferative potential of a compound comprising forming a mixture of ubiquitin; an E1, and E2, and a POSH polypeptide and adding a test agent to determine ubiquitin ligase activity.

Group 73, Claims 73-79, drawn to the special technical feature of a diagnostic method for detecting a neoplastic condition in a subject comprising determining the level of POSH expression in the subject.

The inventions listed as Groups 1-73 do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The technical feature linking Groups 1-73 appear to relate to various POSH polypeptides, methods of screening said polypeptides, therapeutic versions of said polypeptides, inhibitors of said polypeptides, methods of treating neoplastic conditions, and methods of diagnosing neoplastic conditions employing said polypeptides. For example, Claim 1 is drawn to a method for identifying an

INTERNATIONAL SEARCH REPORT

PCT/US03/08194

antiproliferative agent comprising: providing a POSH polypeptide and a test agent; and identifying a test agent that interacts with the POSH polypeptide.

However, Tapon *et al.* (The EMBO Journal. Vol. 17. No. 5. 1998, pp 1395-1404) teaches recombinant expression of a POSH polypeptide with a test agent, and identifying the test agent (Rac) that interacts with the POSH polypeptide.

Therefore, the technical feature linking the inventions of Groups 1-71 does not constitute a special technical feature as defined by PCT Rule 13.2 as it does not define a contribution over the prior art. please See Continuation Sheet

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: POSH NUCLEIC ACIDS, POLYPEPTIDES AND RELATED METHODS

(57) Abstract: The application discloses novel polypeptides and nucleic acids involved in a variety of biological processes, including cellular proliferation. Related methods and compositions are also described.

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